PURIFICATION AND PROPERTIES OF HUMAN HEXOKINASE

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by

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To my son, Aris.

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Abstract

Preliminary attempts were made to isolate hexokinase from human erythrocytes and placenta. The final purifications were made from heart, which contained more enzyme than other tissues. From 1.15 kg of heart, 25 mg of hexokinase was obtained of specific activity 58 units/mg. This represents a 1,700-fold purification and a yield of 47% over six steps. A critical development in isolating this enzyme was the use of affinity chromatography, using glucosamine linked to Sepharose.

The material contained a single protein component according to electrophoresis on starch gel, cellulose acetate and polyacrylamide gel. It gave a single peak on gel filtration, and on sedimentation in the ultracentrifuge.

However, a heterogeneity in size was indicated by electrophoresis on SDS-polyacrylamide gels which gave two bands of approximately equal intensity. The apparent molecular weights of these components were 109,000 and 124,000. The small difference in size has not resolved by the other techniques used.

Gel filtration on Sephadex G-200 suggested a molecular weight of 106,000, as did the combination of the sedimentation coefficient of 5.5 S and diffusion coefficient of 47 $\mu m^2/s$.

Hexokinase takes part in an association equilibrium with polymers of large size as shown by the concentration gradients in sedimentation equilibrium experiments. It is difficult to determine the species involved in this equilibrium as the average molecular weights observed at the meniscus of the solution varied from 110,000 to 140,000.

Study of the kinetic properties of hexokinase gave an optimum at pH = 8.0, I = 0.05. However, the usual kinetic parameters were more reliably measured at pH 7, and gave a Km for glucose of 51 μM and for $MgATP^{2-}$ 0.32 mM.

The usual inhibitor of hexokinase, glucose 6-phosphate, caused an association to probably dimers. This phenomenon was investigated, as it provides a possible means of control (by association or conformational change) on the properties of hexokinase.

Contents

Purchase of materials

(i) For assays

ATP, NADP and NADH from P-L Biochemicals, Inc (USA). TES and Tris from Sigma (London). Yeast G6PD from Boehringer (London).

(ii) For purifications

DEAE Sephadex A-50, CM Sephadex C-50, Sephadex G-200 and Sepharose 4B from Pharmacia (London). DEAE Bio-Gel A and CM Bio-Gel A from Bio-Rad Laboratories (London). Phosphocellulose P-11 from Whatman (Kent, England).

EDTA (B.D.H.), 2-mercaptoethanol (Koch-Light Laboratories Ltd.) Triethanolamine (Fisons Ltd.), PMSF (Sigma, London), 6-aminohexanoic acid (Aldrich Chem. Ltd.), α-D-glucosamine hydrochloride (Hopkin and Williams; Essex), 1-ethyl-3(3-dimethylamino-propyl)carbodiimide hydrochloride (Calbiochem Ltd.)

(iii) For electrophoresis

Acrylamide monomer, bisacrylamide monomer, riboflavin, TEMED, ammonium persulphate and potassium metabisulphite from B.D.H. MTT Tetrazolium, PMS, S.D.S. and periodic acid from Sigma (London). Sudan black B and coomassie brilliant blue R from Difco. Hydrolysed starch (Connaught Lab. Ltd., Canada), Sepharose III electrophoretic strips (Gelman Hawkesly Ltd., Lancing, Sussex), Naphthalene black (I.C.I.), Bromo-phenol blue (Koch-Light Lab. Ltd.), Basic fuchsin (Gurr Ltd., London).

(iv) Protein markers

Cytochrome c, serum albumin, pig heart and rabbit muscle LDH, β-galactosidase, phos-phorylase a and pyruvate kinase from Boehringer (London). Ovalbumin and γ-glob-ulins from Sigma. Myosin from rabbit skelet-al muscle was a gift by Dr G. Offer (Kings College, Department of Biophysics).

(v) Other chemicals

Disodium salts of glucose 6-phosphate and fructose 6-phosphate from Boehringer (London).

Abbreviations

Cyt c	Cytochrome c
CM	Carboxymethyl
DEAE	Diethylaminoethyl
EDTA	Ethylene diamine tetraacetic acid
G6PD	Glucose 6-phosphate dehydrogenase
HK I - IV	Hexokinase types I - IV
LDH	Lactate dehydrogenase
MTT	3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide
PAGE	Polyacrylamide gel electrophoresis
PMS	Phenazine methosulphate
PMSF	Phenylmethyl sulfonylfluoride
SDS	Sodium dodecyl sulphate
TEMED	NNN:N'-Tetraethylene methylene diamine
TES	(N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid)
Tris	Trihydroxymethyl-aminoethane.
V	Volt
A	Ampere
I	Ionic strength
Km	Michaelis constant

Vmax	Maximum velocity
s	Sedimentation coefficient
D	Diffusion coefficient
$\bar{U}$	Partial specific volume
ρ	Density
R	Universal gas constant
k	Boltzman constant
T	Absolute temperature
w	Angular velocity
η	Viscosity.

SECTION 1

Introduction

CHAPTER 1

Mammalian hexokinases

(a) Isoenzymes

Hexokinase is known to exist in four esoenzymic forms in a variety of mammalian tissues. In the rat liver where all four isoenzymes are present they are characterized either as hexokinase types I, II, III and IV in the sequence of increasing electrophoretic mobility (Grossbard and Schimke, 1966) or as A, B, C and D according to their elution from DEAE-cellulose columns (González et al., 1964).

For the correlation of the rat liver isoenzymes with the isoenzymes found in other mammalian species, the following properties are usually considered. Hexokinase types I, II and III have low Km for glucose (E.C. 2.7.1.1) and hexokinase type IV has high Km for the same substrate and is named glucokinase (E.C. 2.7.1.2). Hexokinase type III is inhibited at high glucose concentrations (González et al., 1964; Grossbard and Schimke, 1966). Hexokinase type II is discriminated from the type I by its faster relative mobility on starch gel electrophoresis, its later

elution from DEAE-cellulose chromatography or its greater lability to heat inactivation in the absence of glucose (Grossbard and Schimke, 1966). A summary of the properties of the four hexokinase isoenzymes of rat (Schimke and Grossbard, 1968) is given in Table 1.1.

The hexokinase isoenzymic profile (i.e. types of isoenzymes present and their relative proportions) of mammalian tissues is not constant. Tissue specific differences within a certain species and species-specific differences for a certain tissue are reported. Furthermore developmental, nutritional and hormonal factors were shown to influence the isoenzymic profile of some tissues. Also one hexokinase isoenzyme differs in structure (as shown by amino acid composition, electrophoretic and chromatographic studies) and function (as shown by study of catalytic properties) between different species. However these differences are minor as compared with differences between distinct hexokinase isoenzymes. One type of hexokinase appears rather unaltered in the various tissues of a single species. Certain examples of these general conclusions are presented below.

Rat brain and kidney contain mainly HK I while skeletal muscle contains mainly HK II. Fat pad, intestine and heart contain about equal amounts of HK I and HK II. Kidney and intestine appeared to have also detectable

Table 1.1. Comparison of properties of hexokinases from rat tissues. (After Schimke and Grossbard, (1968)

Electrophoresis	I	II	III	IV
Organs in which present	All organs tested	All organs tested	Liver, kidney small intestine, adrenals - traces in all tissues	Liver only
DEAE-cellulose column elution (M KCl)	0.15	0.22	0.27	0.31
Hexose specificity	Broad	Broad	Broad	Narrow
Km D-glucose (M)	4.5×10^{-5}	2.3×10^{-5}	7.0×10^{-6}	1.2×10^{-2}
Km D-fructose (M)	3.4×10^{-3}	3.4×10^{-3}	3.4×10^{-3}	~2
Vmax fructose/glucose	1.1	1.2	1.2	~5
Nucleotide specificity	ATP ⋙ ITP	ATP ⋙ ITP	ATP ≫ ITP	ATP ⋙ ITP
Km ATP (M)	4×10^{-4}	7.8×10^{-4}	9.8×10^{-4}	5×10^{-4}

cont./

Glucose 6-phosphate inhibition				
a) Versus glucose (Ki) (M)	2.1×10^{-4}	1.6×10^{-4}	9.2×10^{-4}	6.5×10^{-2}
b) Versus ATP (Ki) (M)	2.6×10^{-5}	2.3×10^{-5}	7.4×10^{-5}	
Molecular weight	96,000	96,000	96,000	50,000-60,000
pH optimum	7.8-8.8	7.8-8.8	7.8-8.8	7.5-8.5
Stability	Stable	Unstable	Intermediate	Unstable, stabilized by glucose in presence of 0.1 M KCl.

amounts of HK III, while liver contains all four isoenzymes (Schimke and Grossbard, 1968).

With man, HK I was found in all tissues tested (i.e. heart, erythrocytes, placenta, liver, lung, spleen, kidney, skeletal muscle and fat) while HK II was found in skeletal muscle (Neumann et al., 1974; Rogers et al., 1975b). HK II was also found in erythrocytes (Rogers et al., 1975b) and occasionally in liver (Brown et al., 1967). HK III was found in liver (Rogers et al., 1975b; Neumann et al., 1974; Brown et al., 1967), in lung, spleen, erythrocytes (Neumann et al., 1974) and white blood cells (Rogers et al., 1975b; Povey et al., 1975). HK IV was found in liver (Brown et al., 1967) and brain (Bachelard, 1967b).

Similarities in the tissue distribution of hexokinase isoenzymes, by starch gel electrophoresis, for various mammalian species were shown by Grossbard et al., (1966).

Developmental changes of the isoenzymic profile of tissues are reported for several mammalian species, i.e. guinea pig (Faulkner and Jones, 1976), rat (Katzen and Schimke, 1965), rabbit (Dean and Gusseck, 1976) and man (Holmes et al., 1967; Malone et al., 1968; Schröter and Tillman, 1968; Rogers et al., 1975b). In rat, liver hexokinase types I to IV showed their maximal levels of activity at different postnatal times (Ureta, 1975; Ureta et al., 1975).

With man, HK II appears specifically in newborn's erythrocytes (Holmes et al., 1967) and in foetal liver, heart, brain and muscle (Rogers et al., 1975b). Also in adult human erythrocytes two bands of HK I were observed and the relative activity of these bands changed during maturation of the cells (Kaplan and Beutler, 1968; Rogers et al., 1975a; Rijksen and Staal, 1977).

The effect of nutritional and hormonal factors on the isoenzymic profile of some tissues is well documented. Glucokinase disappears preferentially during starvation or diabetes and reappears upon refeeding with a carbohydrate rich diet or insulin administration probably by "de novo" synthesis (Sharma et al., 1963; Niemeyer et al., 1963; Salas et al., 1963; Clark-Turri et al., 1974). Glucagon and epinephrine are reported to be powerful inhibitors of glucokinase induction (Niemeyer et al., 1966; Ureta et al., 1970).

HK II in insulin-sensitive tissues (including heart) diminishes in diabetes (McLean et al., 1966; Katzen, 1966) and starvation (Katzen and Schimke, 1965), while it increases preferentially upon refeeding (Moore et al., 1964) or by insulin administration (Borrebaek, 1966).

Certain diseases are connected with abnormal isoenzymic patterns or low levels of hexokinase activity. Thus HK II was observed in seven malignant tissues whose

normal counterparts either did not possess this isoenzyme or had it in low levels (Kamel and Schwarzfischer, 1975). Also HK II appeared specifically in erythrocytes of adults with hereditary persistence of foetal haemoglobin (Holmes et al., 1967). A replacement of HK IV by HK II was observed with rat (Sato et al., 1969) and human (Balinsky et al., 1973) hepatomas. Hemolytic anaemias connected with hexokinase deficiency are also reported (Valentine et al., 1967; Keitt, 1969; Gilsanz et al., 1978; Beutler et al., 1978; Board et al., 1978).

The four hexokinase types seem to represent the translation of four separate genes as suggested by various results. Hexokinase isoenzymes are not polymeric forms, differ in their kinetic properties and immunogenic response and present different ontogenetic patterns.

Antiserum against rat brain HK I did not crossreact with HK II and HK III from the same species (Ouchi and Ishibashi, 1975). Also lack of crossreactivity was observed between human heart HK I and spleen HK III with antiserum against HK III and HK I respectively (Neumann et al., 1974; Neumann and Pfleiderer, 1974). Antibodies prepared against rat liver glucokinase did not crossreact with isoenzymes I, II, III whereas they crossreacted with HK IV from other mammalian species (Pilkis and Hansen, 1968; Clark-Turri et al., 1974).

Amino acid composition of HK I from brain (Schwartz and Basford, 1967; Chou and Wilson, 1972) and heart (Easterby and O'Brien, 1973), HK II from skeletal muscle (Holroyde and Trayer, 1976) and glucokinase (Holroyde et al 1976b) show striking similarities leading to the speculation that low Km hexokinases have arisen from an ancestral glucokinase-like gene by gene duplication and divergent evolution (Easterby and O'Brien, 1973; Holroyde and Trayer 1976).

Genetic polymorphism is rather restricted. A rare allele of HK III was found in human leucocytes (Povey et al., 1975). Four alleles of HK I were reported to exist for the primate Macaca irus (Ritter et al., 1974). However screening of erythrocyte lysates of 800 unrelated Europeans revealed no genetic variant for HK I and HK II (Rogers et al., 1975b). Also no polymorphism was observed with rather small samples (20-50 individuals) of heart tissue from man and two monkey species (Ritter et al., 1974).

(b) Subcellular distribution

The appearance of hexokinase in the soluble and particulate states is a widespread phenomenon in mammalian species, initially observed by Crane and Sols (1953).

In many cases particulate hexokinase was established

as mitochondrial i.e. for rat tissues (Wilson, 1967; Vallejo et al., 1970; Mayer and Hübscher, 1971; Wilson and Felgner, 1977), for ascites tumor (Rose and Warms, 1967; Saito and Sato, 1971), for pig heart (Font et al., 1975), for calf brain (Vallejo et al., 1970) and small intestine of guinea pig (Mayer and Hübscher, 1971). In other cases hexokinase was shown to bind both to mitochondria and microsomes as for rat tissues (Spydevold and Borrebaek, 1968; Katzen et al., 1970) or to plasma membranes of hepatomas but not to plasma membranes of normal liver (Emmelot and Bos, 1966; Davidova et al., 1968) and to plasma membranes and microsomes of Ehrlich ascites carcinoma cells (Kang and Coe, 1976).

The percentage of the total hexokinase activity found in soluble and particulate states is a function of animal species and of tissue type. It also depends on the types of hexokinase isoenzymes present and the extraction method used. Some examples of these general considerations are given below.

The tissue-specific subcellular distribution of hexokinase was shown by fractionation of rat tissue homogenates. The ratio of soluble to particulate hexokinase was found to be 80:20 for lung, 55:45 for small intestine, 75:25 for heart and 25:75 for brain while no particulate activity was found in liver (Wilson and Felgner, 1977).

Species-specific differences of subcellular distribution of hexokinase are also reported. In heart of rat, dog and guinea pig most of hexokinase activity was in the soluble state, whereas the opposite was observed with rabbit heart (Mayer et al., 1966).

HK III (Katzen et al., 1970) and HK IV (Wilson and Felgner, 1977) were found only in the soluble form whereas Berthillier et al (1970) reported the binding of rat glucokinase to the microsomal fraction. Hexokinase types I and II were found both in the soluble and particulate states. In one case it was reported that the relative proportions of HK I and HK II were the same in both soluble and particulate states (Mayer and Hübscher, 1971).

Most of the evidence points towards the location of bound hexokinase on the external surface of the outer mitochondrial membrane (Craven et al., 1969; Kropp and Wilson, 1970; Mayer and Hübscher, 1971; Font et al., 1975). Limited evidence is also provided for the binding of hexokinase to the outer surface of the inner mitochondrial membrane or in the intermembrane space (Vallejo et al., 1970; Mayer and Hübscher, 1971).

Electrostatic (Rose and Warms, 1967) and hydrophobic forces have been suggested to be involved in the binding (Teichgräber and Biesold, 1968). Wilson (1973a) suggested the involvement of phospholipid bound on hexokinase for the

interaction of the enzyme with the mitochondria.

Solubilization of the particulate hexokinase was achieved by glucose 6-phosphate (Hernandez and Crane, 1966; Rose and Warms, 1967; Wilson, 1968), nucleotides (Rose and Warms, 1967; Wilson, 1967, 1968) and high concentrations of salt (Hernandez and Crane, 1966; Rose and Warms, 1967; Teichgräber and Biesold, 1968; Easterby and O'Brien, 1973). Solubilization mediated by the above agents was pH-dependent, as was the solubilization in the absence of these agents (Rose and Warms, 1967; Teichgräber and Biesold, 1968; Easterby and O'Brien, 1973, Aleksakhina et al., 1973).

Solubilization by ATP and glucose 6-phosphate was found relatively specific compared to that by high concentration of salt (Wilson, 1968). Inorganic phosphate was found to reverse glucose 6-phosphate mediated solubilization (Rose and Warms, 1965, 1967; Wilson, 1968) but had no effect by itself or on ATP mediated solubilization (Wilson, 1968). Magnesium ions at 1 mM concentration were found to suppress glucose 6-phosphate-mediated elution from mitochondria (Rose and Warms, 1967; Wilson, 1968), but had no effect on ATP mediated solubilization even at higher concentrations (Wilson, 1968). The fact that the combined effect on solubilization of ATP and glucose 6-phosphate was less than their individual effect indicated that they are

not acting independently (Wilson, 1968).

Several results point towards a dynamic equilibrium between the soluble and particulate bound hexokinase. Rose and Warms (1967) suggested that ascites tumor hexokinase interaction with mitochondria follows a simple over-all equation i.e. (binding sites) + (enzyme) + (Mg^{2+}) $\rightleftharpoons$ (complex). Hernandez and Crane (1966) observed that approximately a constant proportion of porcine heart hexokinase was solubilized with successive extractions and that the eluted enzyme could be rebound to mitochondria. However Thompson and Bachelard (1977) suggested on kinetic evidence non-identity between the soluble and bound or solubilized brain hexokinase.

Latent hexokinase (bound so as to render itself inaccessible to exogenous substrate) seems to be mainly connected with hexokinase type I in heart (Katzen et al., 1970) and brain (Wilson, 1967) and was exposed and extracted by glucose 6-phosphate and ATP (Bachelard, 1967a; Wilson, 1967, 1968), salts (Teichgräber and Biesold, 1968), osmotic shock (Wilson, 1967; Kropp and Wilson, 1970), rapid freeze thawing (Wilson, 1967, 1968) and detergents such as Triton X-100 (Wilson, 1967; Kropp and Wilson, 1970).

In rat heart, HK I was unaffected by diabetes but HK II decreased to an equal extent in both soluble and particulate forms, (Katzen et al., 1970). In rat mammary

glands diabetes decreased particulate hexokinase from 46% of the total enzyme in the cell to 11%, while the activity of the soluble fraction was not significantly altered (Walters and McClean, 1967). On the other hand intraperitoneal injection of insulin increased by a factor of 3 the hexokinase type II bound to hepatic mitochondria in a very short time (Bessman and Gots, 1975).

(c) Purifications

A summary of purifications of low Km mammalian hexokinases which include estimates of homogeneity is presented in Table 5.3.

HK I

The main problem in the purification of mammalian hexokinases is the low level of enzyme-activity in the tissues. It is understandable therefore why the first successful purifications dealt with HK I solubilized from brain mitochondria. Brain contains only HK I mainly bound to mitochondria. Elution of the enzyme from mitochondria by detergent, salts or glucose 6-phosphate offered a starting material that needed only 10 to 100-fold further purification for homogeneity.

Schwartz and Basford (1967), purified bovine brain hexokinase (homogeneous by sedimentation velocity ultra-

centrifugation and electrophoresis) by solubilization of the mitochondrial enzyme by Triton X-100 and 10-fold extra purification by salt fractionation and two anion-exchange chromatographic steps. The specific activity of the purified material was 80 units/mg but the yield was very low (1.5%). However this preparation is suspicious since mitochondria were pretreated with chymotrypsin prior to the elution of hexokinase.

Redkar and Kenkare (1972) purified the same enzyme to a specific activity of 83 units/mg by a similar procedure to that of Schwartz and Basford (1967) with one notable change i.e. hexokinase was eluted from mitochondria by NaCl. The homogeneity of their preparation was tested by sedimentation velocity ultracentrifugation and electrophoresis.

The same year Chou and Wilson (1972) reported an elegant purification of rat brain hexokinase by specific solubilization of the mitochondrial enzyme by glucose 6-phosphate and only 3-fold extra purification by anion exchange chromatography. The purified material had a specific activity of 60 units/mg and the homogeneity was tested by several methods including isoelectric focussing, sedimentation equilibrium ultracentrifugation and SDS-PAGE.

Easterby and O'Brien (1973) purified HK I from porcine heart by salt solubilization ($MgCl_2$) at low pH of the mito-

chondrial enzyme, ion and cation exchange chromatographic steps, and gel filtration. The purified material had a specific activity of 82 units/mg and was extensively tested for homogeneity (Easterby, 1971; Easterby and O'Brien, 1973).

HK II

HK II was purified (in a similar fashion to glucokinase) by Holroyde and Trayer (1976) from rat skeletal muscle to a particularly high final specific activity of 210 units/mg. The homogeneity of this purified hexokinase was tested by high speed sedimentation equilibrium and SDS-PAGE. Contamination of this preparation with HK I can not be excluded since hexokinase type I was found by Grossbard and Schimke (1966) to represent a small fraction of rat skeletal muscle hexokinase activity.

HK IV

Glucokinase was purified from rat liver by Grossman et al., (1974) by salt fractionation, anion exchange chromatographic steps and gel-filtration to a final specific activity of 80 units/mg. The enzyme was found homogeneous by low protein load SDS-PAGE. However the yield was very low (2.4%).

The same enzyme was purified to high yield by Holroyde

et al., (1976b) by anion exchange chromatographic steps, gel filtration and affinity chromatography. These authors incorporated for the first time a specific chromatographic step (glucosamine immobilized on Sepharose 4B) for the purification of a hexokinase. The final specific activity of the purified material was 150 units/mg and the homogeneity of the material was vigorously tested.

Human hexokinases

Hexokinases from various species and tissues have been purified to various levels of purity. They are not mentioned here since even the best of them are not supported by information concerning the homogeneity of the purified enzymes. However it is interesting to refer to such purifications of the enzyme from human tissues.

Erythrocyte hexokinase was purified by Gerber et al., (1974) and Rijksen and Staal (1976a) (Table 3.2). The best of these purification procedures (Rijksen and Staal, 1976a) gave 0.4 mg of purified material of 14 units/mg specific activity, by the use of salt fractionation, anion exchange chromatographic steps, gel filtration and affinity chromatography (glucosamine immobilized on Sepharose 4B).

Neumann et al., (1974) purified HK I from heart by salt fractionations, anion exchange chromatographic steps and gel filtration to a final specific activity of 71 units/

mg. The yield was very low (2.5%). Using a similar procedure on a small scale these authors purified HK III from spleen to a final specific activity of 3 units/mg. This is the best attempt to purify HK III from mammalian tissues.

(d) Molecular size

A summary of the molecular weights of mammalian hexokinases reported for the native and denatured enzyme is presented in Table 1.2.

A general conclusion is that low Km hexokinases have molecular weight of about 100,000 while glucokinase has a molecular weight of about 50,000. Although rare cases of subunit structure of low Km hexokinases have been reported (Monakhov et al., 1973; Craven and Basford, 1974), the bulk of the research shows that hexokinase types I, II and IV are composed of a single polypeptide chain (HK III has not yet been tested).

However the presence of particularly strong interactions between subunits can not be ruled out, but such a possibility seems remote because a single band corresponding to a molecular weight of about 100,000 was observed by the denaturing conditions of SDS polyacrylamide gel electrophoresis. Also a single N-terminal amino acid, glycine, was found for rat brain hexokinase representing 0.8 moles per 98,000 g (Chou and Wilson, 1972).

Table 1.2. Molecular weights of mammalian hexokinases

The following abbreviations, for the methods of estimation of molecular weight, are used below.

G.F. : gel filtration ; D.G. : Density gradient ultra-centrifugation ; S.V. : Sedimentation velocity ; S.E. : Sedimentation equilibrium ; S.D.S. : SDS-poly-acrylamide gel electrophoresis.

Tissue	Molecular weight x 10^3	Method of estimation of M. wt.	Reference
Rat brain (HK I)	96	D.G.	Grossbard and Schimke (1966)
Rat brain	98	D.G.	Wilson (1972)
Rat brain	98	D.G.	Chou and Wilson (1972)
Rat brain	130→ 100→ 45	S.D.S.	Craven and Basford, (1974)
Rat brain	96 - 98	S.D.S.	Chou and Wilson (1972)
Rat brain	97.5 ± 0.5	S.E.	Chou and Wilson (1972)
Rat muscle	96	D.G.	Grossbard and Schimke (1966)
Rat muscle	98	S.D.S.	Holroyde and Trayer (1976)
Rat muscle	96	S.E.	Holroyde and Trayer (1976).

cont./

Tissue	Molecular weight x 10^3	Method of Estimation of M.wt.	Reference
Rat kidney (HK I)	99	D.G.	Grossbard and Schimke (1966)
Rat liver (HK III)	99.5	D.G.	Grossbard and Schimke (1966)
Rat liver (HK IV)	55 ⇌ 120	G.F.	Berthillier and Got (1972)
Rat liver (HK IV)	49.5	G.F.	Holroyde et al. (1976b)
Rat liver (HK IV)	52	S.D.S.	Holroyde et al. (1976b)
Rat liver (HK IV)	48.6	S.E.	Holroyde et al. (1976b)
Rat liver (HK IV)	47	G.F.	Pilkis (1972)
Rat liver (HK IV)	57	G.F.	Grossman et al. (1974)
Rat liver (HK IV)	53	S.D.S.	Grossman et al. (1974)
Rat liver (HK IV)	58 ⇌ 110	G.F.	Niemeyer et al. (1975)
Rat liver (HK IV)	48 - 65	G.F.	Pilkis et al. (1968)
Rat liver (HK IV)	68	D.G.	Pilkis et al. (1968)
Rat fat pad (HK II)	90	D.G.	Grossbard and Schimke (1966)
Porcine heart	97.5	S.D.S.	Easterby (1971)
Porcine heart	96	S.E.	Easterby (1971)
Bovine brain	107	G.F.	Redkar and Kenkare (1972).
Human erythrocytes	132	G.F.	Rijksen and Staal (1976a)
Human erythrocytes (HK I)	104	G.F.	Rogers et al. (1975b)
Human erythrocytes (HK II)	108.4	G.F.	Rogers et al. (1975b)

cont./

Tissue	Molecular weight x 10^3	Method of Estimation of M.wt.	Reference
Human heart (HK I)	111	G.F.	Neumann et al. (1974)
Human spleen (HK III)	116	G.F.	Neumann et al. (1974)
Human fetal liver (HK I)	103.9	G.F.	Rogers et al. (1975b)
Human fetal liver (HK III)	107.2	G.F.	Rogers et al. (1975b)
Human gastric mucosa (Four iso-enzymes)	112 113 118 125	D.G.	Monakhov et al. (1973) Monakhov et al. (1975)
Human gastric mucosa (Four iso-enzymes)	58 - 60	S.D.S.	Monakhov et al. (1973) Monakhov et al. (1975)

Hexokinase isoenzymes types are assigned according to the references quoted.

Polymerization of hexokinases was observed in certain cases.

Rat glucokinase occasionally was observed, by gel filtration experiments, to exist as species of 58,000 and 110,000 molecular weight, suggested as monomer and dimer respectively (Niemeyer et al., 1975). By gel filtration microsomal rat hepatic glucokinase was found to exist as monomer and dimer of 55,000 and 120,000 molecular weight, with ATP favouring the associated form (Berthillier and Got, 1972).

The presence of small amounts of polymeric forms of hexokinase were observed for porcine heart HK I by electrophoresis, ion-exchange chromatography and low speed sedimentation equilibrium experiments (Easterby, 1971, 1975; Easterby and O'Brien, 1973) and for bovine brain HK I by sedimentation velocity experiments (Chakrabarti and Kenkare, 1974).

For both the above enzymes the presence of glucose 6-phosphate greatly enhanced the association to dimers. Thus in the presence of 1 mM glucose 6-phosphate, and at a protein concentration of 2-3 mg/ml, dimers accounted for 50% for the porcine heart enzyme (Easterby, 1975) and 95% for the bovine brain enzyme (Chakrabarti and Kenkare, 1974) of total hexokinase, as shown by integration of sedimentation boundaries.

The glucose 6-phosphate mediated dimerization thus monitored was found in both cases to be reversible. Maximal extent of dimerization was reached at 50 μM glucose 6-phosphate concentration for the porcine heart hexokinase and at 100 μM for the bovine brain enzyme. At the low protein concentration of 1 mg/ml, no dimerization was observed for the bovine brain enzyme.

Several metabolites modulated the glucose 6-phosphate mediated dimerization in a parallel way to their effect on the catalytic properties of the enzyme, although activity measurements were performed at much lower enzyme concentrations than in the ultracentrifuge experiments. Thus ATP alone and to a greater extent $MgATP^{2-}$, and Pi reversed the dimerization of procine heart hexokinase at low glucose 6-phosphate concentrations (Easterby, 1975). Also ATP and Pi completely reversed the glucose 6-phosphate mediated dimerization of bovine brain hexokinase (Chakrabarti and Kenkare, 1974). Glucose, for both enzymes, had no effect either in promoting dimerization or relieving glucose 6-phosphate mediated dimerization.

Fructose 6-phosphate and mannose 6-phosphate, non-inhibitors of hexokinase did not promote dimerization of porcine heart hexokinase even at high concentrations, whereas glucose 1,6-diphosphate, a known inhibitor of mammalian hexokinases (Crane and Sols, 1954; Gerber et al.,

1974; Rose and Warms, 1965), produced detectable dimers although at a concentration over 200 μM (Easterby, 1975). Fructose 6-phosphate and ADP did not promote dimerization of bovine brain hexokinase (Chakrabarti, 1974).

The establishment of an equilibrium between monomer and dimer of porcine heart hexokinase in the presence of glucose 6-phosphate was suggested because the ratio of monomer to dimer remained constant during incubation of the enzyme for several days with this metabolite. However, larger polymers were produced in slow process as shown by sedimentation equilibrium experiments (Easterby, 1975).

Rat brain hexokinase was found to aggregate at low ionic strength (lower than 0.05) at pH = 7.0 as seen by increase in turbidity of the enzyme solution (Wilson, 1972). By sucrose density preparative ultracentrifugation the sedimentation coefficient of the same enzyme was found to increase in the presence of 1.5 mM glucose 6-phosphate. A further increase was observed by increasing the protein concentration. The increase in sedimentation coefficient was attributed to some polymerization in the presence of glucose 6-phosphate (Wilson, 1973b).

(e) <u>Catalytic properties</u>

It is beyond the scope of this introduction to give a

full description of the voluminous literature on the catalytic properties of mammalian hexokinases. Therefore observations for human hexokinases are given priority with brief notes for other mammalian hexokinases where relevant.

Hexokinases catalyse the following reaction

$$MgATP^{2-} + \text{hexose} \longrightarrow MgADP^{1-} + \text{hexose 6-phosphate}^{2-} + H^{+}$$

The catalysed reaction is a non-equilibrium one. For Rhesus monkey skeletal muscle hexokinase the apparent equilibrium constant for hexokinase was over 1,000 times larger than the mass-action ratio (Beatty et al., 1976).

Human erythrocyte hexokinase was found specific for MgATP as the nucleotide substrate, whereas MgITP, MgGTP, MgUTP and MgCTP were not converted to their diphosphates at concentrations of 10 mM (Rijksen and Staal, 1976a). The same authors found that mannose was phosphorylated at the same rate as glucose although with a lower affinity. Much lower rates and affinities were found for 2-deoxy-D-glucose, D(+) glucosamine and D(-)fructose. N-acetyl-D-glucosamine, D(+)galactose, D(+)xylose and L(-)sorbose were not phosphorylated at all by human erythrocyte hexokinase. This is in agreement with the observations by Sols and Crane (1954) for the importance of hydroxyl groups at carbon atoms 1, 3, 4 and 6 of hexoses, for the specificity of rat brain hexokinase. A summary of Michaelis constants of human hexokinases for glucose and MgATP is given in Chapter 6.

Crane and Sols (1954) found that although certain hexoses were good substrates for brain hexokinase only glucose 6-phosphate was an inhibitor (non-competitive against glucose) of the enzyme. On this basis they postulated the existence of a specific binding site for glucose 6-phosphate.

Glucose 6-phosphate was observed to inhibit human erythrocyte hexokinase competitively towards MgATP (Ki = 11 μM) but non competitively towards glucose (Gerber et al., 1974; Rijksen and Staal, 1977). Solubilized human placental hexokinase was also inhibited in the same fashion with Ki against MgATP and glucose of 27 and 78 μM respectively (Gustke, 1975). These findings suggest that glucose 6-phosphate binds at a separate site than the hexose binding site in the active center but it shares the MgATP binding site.

With porcine heart hexokinase (Easterby and O'Brien, 1973) competitive inhibition was observed by Glucose 6-phosphate against MgATP (Ki = 20 μM) and non-competitive inhibition against fructose or mannose (Ki = 50 μM). For human erythrocyte hexokinase, fructose 6-phosphate was not found to inhibit the hexokinase reaction by Gerber et al., (1974) whereas Rijksen and Staal (1977) reported competitive inhibition by fructose 6-phosphate against MgATP (Ki = 0.16 mM).

Rijksen and Staal (1976b) reported that Mg^{2+} and ATP^{4-} inhibited human erythrocyte hexokinase non-competitively against glucose but competitively against $MgATP^{2-}$ with Ki equal to 16-18 and 1.6 mM respectively. The observation of Gerber et al., (1974) that Mg^{2+} at low concentration is an activator of the human erythrocyte enzyme may arise from complex formation with ATP^{4-}, which normally inhibits the enzyme (Rijksen and Staal, 1976b).

Contradictory results have been reported for the effect of inorganic phosphate on human erythrocyte hexokinase. Thus Pi has been found to relieve partly (de Verdier and Garby, 1965; Rijksen and Staal, 1977), or completely (Kosow et al., 1973) the inhibition of glucose 6-phosphate, whereas Gerber et al., (1974) found no influence at all.

Ellison et al., (1974) suggested a model for bovine brain hexokinase which implies the existence of an equilibrium between a free and a phosphate-associated enzyme, where glucose 6-phosphate binding to the phosphate-associated form is very poor. Wilson (1973b) proposed a model for brain hexokinase according to which the enzyme exists in a rapid equilibrium between two alternate forms one binding preferentially inorganic phosphate and the other glucose 6-phosphate. Most of the kinetic results for the human erythrocyte enzyme conform with these models.

Inorganic phosphate was found to inhibit human erythrocyte hexokinase competitively against $MgATP^{2-}$ with Ki = 20 mM (Rijksen and Staal, 1977).

MgADP and MgAMP inhibited human erythrocyte hexokinase competitively against MgATP with Ki of 1 and 0.7 mM respectively (Rijksen and Staal, 1977).

Human erythrocyte hexokinase was found to operate by a rapid equilibrium random mechanism (Gerber et al., 1974) in accordance with proposed mechanisms for other mammalian hexokinases (Kosow and Rose, 1968; Bachelard et al., 1971; Purich and Fromm, 1971).

Easterby and O'Brien (1973) suggested that rapid equilibrium random and compulsory order mechanisms are indistinguishable if the first substrate to bind to the enzyme is in equilibrium with its enzyme-substrate complex, concluding that for the porcine heart enzyme at least one of the enzyme substrates is in equilibrium with its enzyme-substrate complex.

(f) Stability

The binding of metabolites to hexokinase, observed by various conformational changes of the enzyme molecule leading to release from mitochondria, polymerization and catalysis has already been mentioned.

Further information of enzyme-ligand interactions has been obtained from assessments of protection (of the enzyme by ligands) against inactivation by heat, proteolysis, sulfhydryl reagents, glutaraldehyde and Ellman's reagent. From the protective effects dissociation constants of enzyme-ligand complexes have been estimated.

According to Redkar and Kenkare (1975), glucose 6-phosphate completely protected bovine brain hexokinase against DTNB, while glucose protected to a lesser extent. Although ATP, MgATP, ADP, MgADP and Pi changed the reactivity of some sulfhydryl groups on the enzyme they did not protect hexokinase against inactivation. Protection by fructose 6-phosphate was similar to that by glucose. These authors interpreted these and other results as indicating that glucose 6-phosphate and inorganic phosphate share the same locus on the enzyme as γ-phosphate of ATP and that nucleotides ATP and ADP bind to the enzyme in the absence of Mg^{2+}.

Similar results were presented by Chou and Wilson (1974) who also studied the interaction of DTNB with -SH groups on the rat brain enzyme, which showed a pronounced conformational change of the enzyme molecule upon binding of glucose 6-phosphate.

Wilson (1978) observed that UTP, ITP, GTP and CTP were much less effective than ATP at protecting rat brain

hexokinase against inactivation by chymotrypsin. With glutaraldehyde only good substrates (glucose, mannose, 2-deoxyglucose) protected, while nonsubstrates (galactose, arabinose, N-acetyl glucosamine) were not effective. Also with glutaraldehyde, glucose 6-phosphate was more effective at protecting the enzyme's molecule than mannose 6-phosphate or fructose 6-phosphate. A model was proposed by this author based on these and his previous observations, taking into account ligand-induced conformations at various points of the catalytic cycle.

The differences in heat and proteolytic inactivation of hexokinase types I to IV were investigated by Grossbard and Schimke (1966) and Salas et al., (1965). HK II and HK IV were found unstable, whereas in the presence of glucose they were stabilized. In addition KCl was needed for the stability of HK IV.

Murakami and Rose (1974) investigated the heat inactivation of hexokinase type II. Glucose at 10 mM protected the enzyme, whereas at lower concentration (0.1 mM) it did not, except if glucose 6-phosphate, Pi or ADP were also present.

(g) <u>Regulation</u>

Hexokinase catalyses an irreversible reaction and to-

gether with phosphofructokinase has been accepted as a key point in the regulation of glycolytic flux in human erythrocytes (Rapoport et al., 1974; Heinrich and Rapoport, 1974).

Hexokinase catalyses the first step of glycolysis, phosphorylating glucose entering in the cell. Its product, glucose 6-phosphate, appears in a central position in the carbohydrate metabolism. It is also derived from glycogenolysis and is chanelled to glycolysis, pentose phosphate pathway, glycogenesis or is returned to glucose via phosphatase action, the direction(s) determined by the metabolism of the tissue involved.

Under intracellular conditions the glucose 6-phosphate concentration in human erythrocytes is such that most of hexokinase is expected to be in an inhibited form (Rose and O'Connell, 1964; Gerber et al., 1974; Rijksen and Staal, 1977) and is therefore strongly regulated by the glucose 6-phosphate level. Also rat skeletal muscle hexokinase was estimated to be 80% inhibited under intracellular conditions, increased to 96% under tetanic muscular contraction (Lueck and Fromm, 1974).

In human erythrocytes, inorganic phosphate, was found to exert its most pronounced influence in relieving glucose 6-phosphate inhibition at concentrations that are physiologically important, so that Pi was suggested to have a regulatory role (Rijksen and Staal, 1977). The same

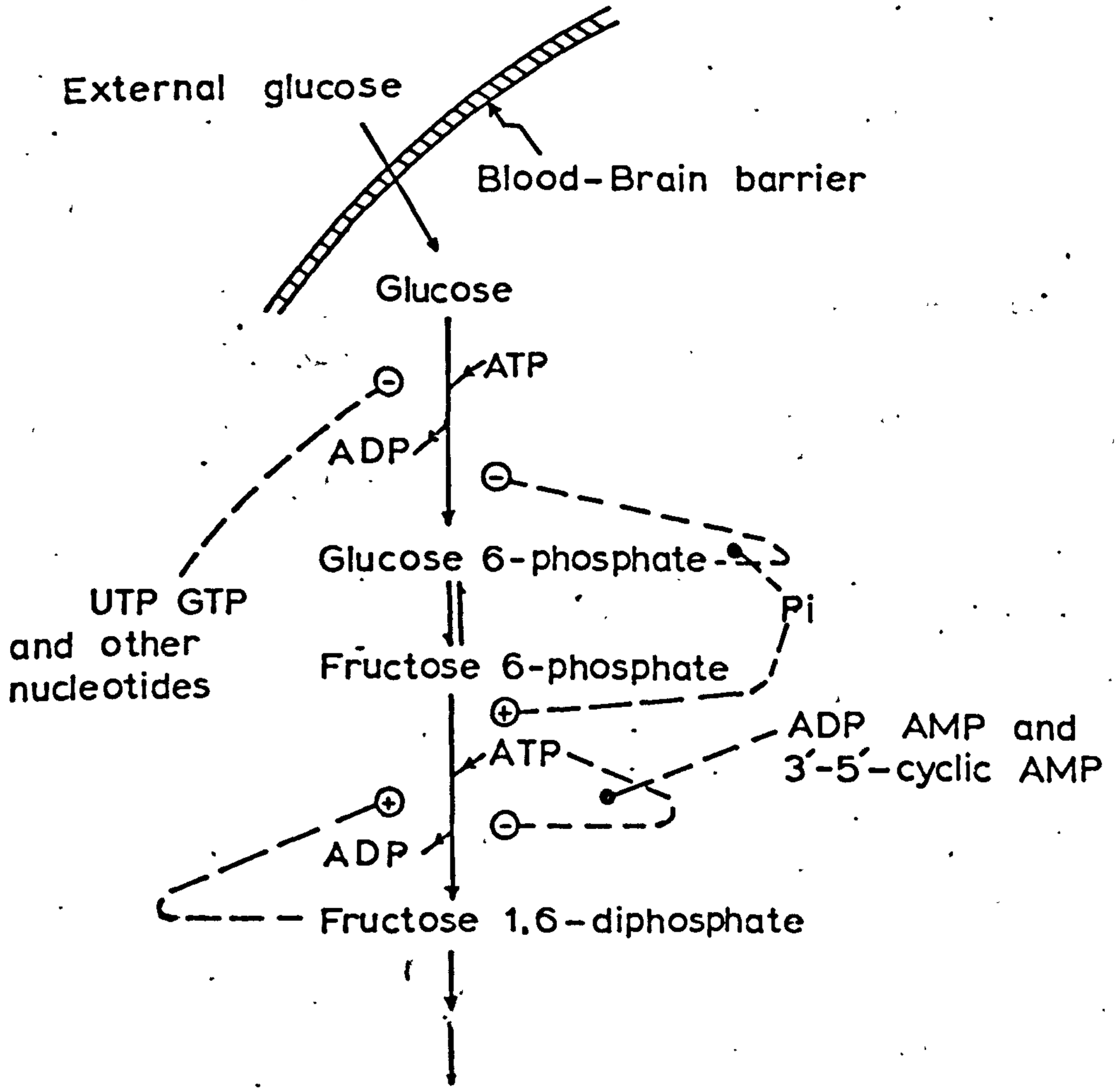
authors suggested that glucose has no regulatory role, since it exists in saturating levels in erythrocytes, while factors affecting the concentration of MgATP (that is near the Km for this metabolite) must be potential regulators too.

Purich et al., (1973) proposed a model for the control of glycolytic flux in brain and erythrocytes at the hexokinase and phosphofructokinase catalysed reactions taking into consideration the effect of glucose 6-phosphate, Pi and nucleotides (Figure 1.1).

The regulation on hexokinase should be exerted at other levels too. Thus the amount and type of hexokinase(s) in the cell determined by synthesis and inactivation of each isoenzyme seem significant. The variations of activity levels of HK II and HK IV with diabetes and starvation, as well as ontogenetic variations fall into this type of regulation. Another factor of regulation seems to be the intracellular localization of hexokinase.

Glucose 6-phosphate had little effect on the activity of the particulate hexokinase from rat brain (Tuttle and Wilson, 1970). Therefore the inhibition by glucose 6-phosphate acts in two steps: the transfer of hexokinase from the particulate to soluble form followed by strong inhibition of the soluble form. Also hexokinase from rat liver had a higher affinity for ATP generated within the

Figure 1.1. Regulation of early reactions in the glycolytic pathway in brain and erythrocytes (after Purich et al., 1973).



⊕ Activation

⊖ Inhibition

• Deinhibition.

mitochondrion than that external to the organelle (Gots et al., 1972; Gots and Bessman, 1974).

Font et al., (1975) interpreting their own and other results pointed out that "the association of hexokinase with mitochondrial membranes may serve to control glucose phosphorylation, especially in muscle and brain; the localization close to the ATP-generating system would efficiently provide ATP, and thus, glucose 6-phosphate in sufficient amounts to ensure metabolism, but high concentrations of glucose 6-phosphate or ATP, solubilize hexokinase and modify its kinetic properties".

Glucokinase serves a specific function. It is not inhibited by physiological levels of glucose 6-phosphate and thus glucose coming from the portal vein (at concentrations near the Km for this isoenzyme) is readily phosphorylated for glycogen synthesis (Newsholme and Start, 1973).

SECTION 2

Purifications

Extensive work has been done in this Laboratory on the purification and physicochemical study of human erythrocyte enzymes as glucose 6-phosphate dehydrogenase (Cohen and Rosemeyer, 1969a, 1969b), 6-phosphogluconate dehydrogenase (Pearse and Rosemeyer, 1974a, 1974b) and glutathione reductase (Worthington and Rosemeyer, 1974, 1975, 1976).

The aim of the present research was the purification and physicochemical study of human erythrocyte hexokinase. Because of the problems involved in the purification of the erythrocyte enzyme (Chapter 3), exploratory small scale purifications were tried with relatively easily available human tissues as placenta (Chapter 4) and heart that contained HK I. The latter tissue proved more favourable and so a big scale purification procedure was devised for hexokinase from this tissue (Chapter 5).

CHAPTER 2

Materials and methods.

(a) Human tissues

Blood, out-dated for transfusion, was obtained from the Blood Bank. It had normally been stored for at least three weeks at 0 - 4°C in acid/citrate/dextrose.

Term placentas were obtained after delivery and stored at -25°C.

Hearts were obtained from autopsy and stored at -25°C, for one week to one month till the desired amount of tissue for a purification was collected.

(b) Buffers

The following buffers were used in the purification and further additions to these are indicated at the appropriate stages. Analytical grade reagents were used where available and pH was measured at room temperature.

Potassium phosphate pH = 8.0, I = 0.1 containing:

0.34 g/l KH_2PO_4

5.66 g/l K_2HPO_4

Potassium phosphate pH = 7.5, I = 0.02 containing:

0.27 g/l KH_2PO_4

1.04 g/l K_2HPO_4

Potassium phosphate pH = 7.0, I = 0.1 containing:

2.74 g/l KH_2PO_4

4.63 g/l K_2HPO_4

Potassium phosphate pH = 6.5, I = 0.05 containing:

3.29 g/l KH_2PO_4

1.50 g/l K_2HPO_4

Potassium phosphate pH = 6.5, I = 0.1 containing:

6.04 g/l KH_2PO_4

3.23 g/l K_2HPO_4

Potassium acetate pH = 5.8, I = 0.05 containing:

4.91 g/l CH_3COOK

0.213 ml/l CH_3COOH

Potassium acetate pH = 5.8, I = 0.025 + 0.125 M KCl containing:

2.45 g/l CH_3COOK

0.112 ml/l CH_3COOH

9.32 g/l KCl

Potassium acetate pH = 5.6, I = 0.1 + 0.2 M $MgCl_2$ containing:

9.81 g/l CH_3COOK

0.257 ml/l CH_3COOH

40.6 g/l $MgCl_2.6H_2O$

20 mM Triethanolamine-HCl pH = 7.0 + 5% glycerol

containing: 2.65 ml/1 Triethanolamine

50 ml/1 glycerol

All buffers contained 0.1% ($\bar{v}$) 2-mercaptoethanol, 1 mM K_2EDTA and 10 mM glucose unless otherwise stated. Solubilization experiments were also performed in the absence of these substances.

(c) Preparation of resins

1. DEAE - Sephadex A-50

This anion exchanger had a particle size of 40-120 µm. The resin was left to swell for two days at room temperature in the appropriate initial buffer. It was washed with several volumes of the same buffer by filtering the suspension on a Buchner funnel and resuspending the cake in fresh buffer. After each washing the pH of the suspension was adjusted to that of the buffer. The resin was considered to be equilibrated when the pH and conductivity of the eluate was the same as the applied buffer.

2. CM - Sephadex C-50

This cation exchanger had a particle size of 40-120 µm. The resin was swelled and equilibrated in the same way as the Sephadex A-50.

3. P-11 cellulose

This cation exchanger was a fibrous cellulose phosphate resin. It was swelled and equilibrated in the same way as the Sephadex C-50 with one addition. Since phosphocellulose had a brownish colour and the buffer used for swelling was becoming coloured, the suspension was washed on a Buchner funnel with several volumes of the same buffer containing 1 M KCl until the eluate was colourless. Subsequent washing of the resin with above buffer alone removed the salt. In this way impurities were removed that otherwise would contaminate the fractions during the elution.

4. Sephadex G-200

This resin used for gel filtration had particle size of 40-120 μm. It was allowed to swell in the buffer used for gel filtration for three days and was poured into the column at room temperature under atmospheric pressure. The column was transferred in a cold room with a temperature of 0 - 4°C and was allowed to stand for 3 to 4 weeks before use, to enable the resin to settle and allow for complete equilibration. This gave better and more reproducible results as was found previously in this laboratory (P. Cohen Ph.D. Thesis, 1969). The same column was used several times until the flow rate was reduced substantially.

After each use the column was washed with buffer containing 0.02% $(\frac{w}{v})$ sodium azide as antibacterial agent. Before use the column was washed with fresh buffer for two days to remove the sodium azide.

5. Sepharose 4B with immobilized glucosamine

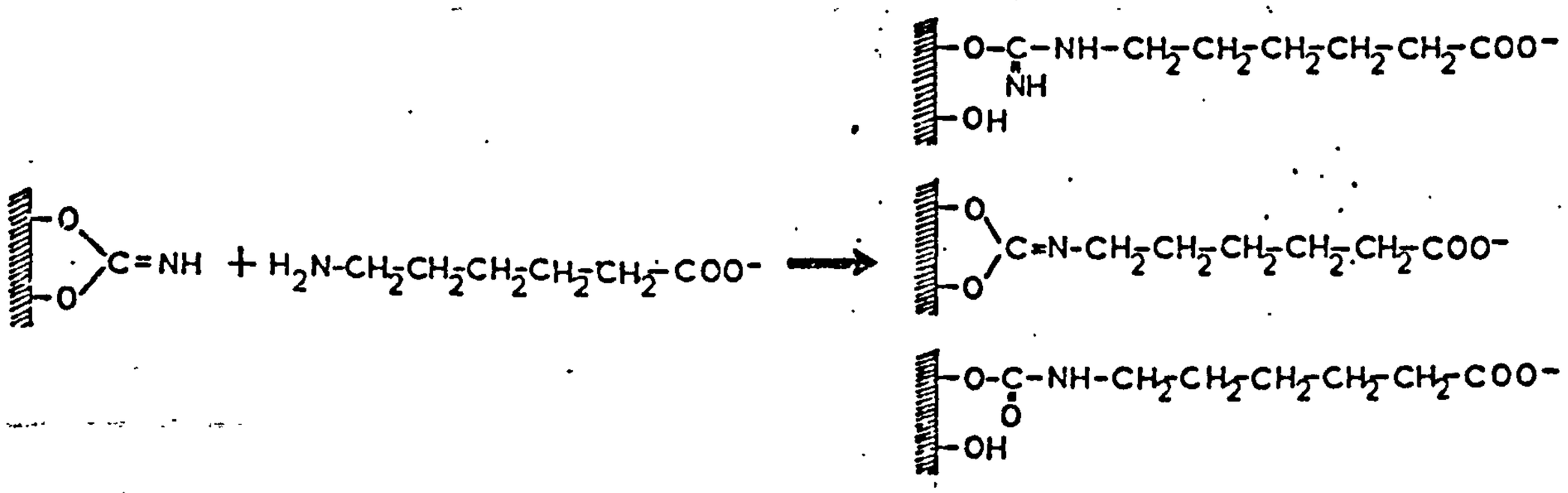
This affinity resin was prepared using Sepharose 4B as supporting matrix, 6-aminohexanoic acid as spacer arm and α-D-glucosamine as ligand. It was prepared in three steps according to the method of Cuatrecasas (1970) with slight modifications. A scheme for the reactions involved in this synthesis is given in Figure 2.1.

(i) Activation of Sepharose 4B by the cyanogen bromide method.

One volume of settled Sepharose 4B was mixed with equal volume of water. In the suspension cyanogen bromide was dissolved to a final concentration of 65 mg per ml of settled Sepharose 4B. The reaction temperature was kept at 20 - 25°C by the addition of crushed ice and continuous stirring. The pH was maintained at 11.0 with the addition of 5N NaOH. After 15 minutes the reaction was complete as judged by the constancy of pH. The suspension was filtered and quickly washed with 15 volumes of chilled 0.1 M sodium carbonate buffer pH = 10.0. Free cyanide ions were treated with excess of sodium hypochlorite for several hours before

(i) Activation of Sepharose with Br-CN.

(ii) Coupling of 6-aminohexanoic acid to activated Sepharose.



(iii) Coupling of glucosamine to Sepharose-N-6-aminohexanoic acid by a carbodiimide promoted reaction.

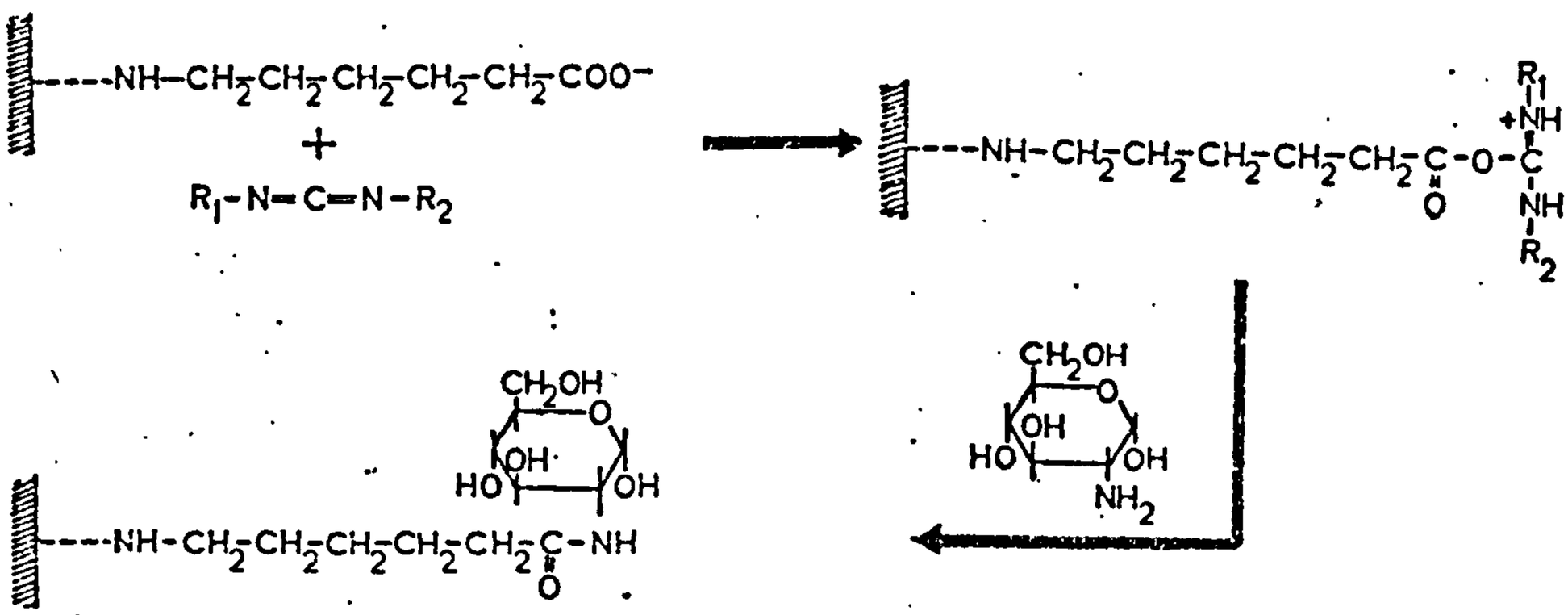


Figure 2.1. Reactions involved in the synthesis of Sepharose-N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose.

the disposal of washings.

(ii) Coupling of 6-aminohexanoic acid to the activated Sepharose 4B.

The activated Sepharose 4B cake was immediately suspended in two volumes of 0.1 M sodium carbonate buffer pH = 10.0 containing 1.7 mmoles of 6-aminohexanoic acid per ml of settled Sepharose 4B. The suspension was left overnight under continuous stirring at $4^{o}C$. Subsequently it was filtered and washed successively with 10 volumes of water, 7 volumes of 1 M KCl in water and 15 volumes of water.

(iii) Coupling of α-D-glucosamine to Sepharose-N-6-aminohexanoic acid.

The resin was suspended in two volumes of water containing 70 μmoles of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride and 0.4 mmoles of α-D-glucosamine hydrochloride per ml of settled Sepharose. The reaction lasted for 3 hours with continuous stirring at room temperature while the pH was maintained at 4.75. The suspension was filtered and the resin was successively washed with 7 volumes of water, 7 volumes of 2M KCl in water and 7 volumes of the appropriate buffer for the affinity chromatography.

For storage at $4^{o}C$, sodium azide was added as anti-

bacterial agent to a final concentration of 0.02% ($\frac{w}{v}$) to the suspension. Before use the resin was washed with fresh buffer for the removal of sodium azide. After use the resin was washed with 5 volumes of 2 M KCl/6 M Urea in water for the removal of any firmly bound proteins on the resin, as reported by Holroyde et al. (1976 a).

6. Sepharose 4B with immobilized N^6-[(6-aminohexyl)carbamoylmethyl]-ATP

The ATP analogue (Figure 2.2) was a gift from Dr K. Mosbach. It's synthesis is described by Lindberg and Mosbach (1975) and it's coupling to Sepharose 4B was made according to this reference with slight modifications, in two steps.

(i) Activation of Sepharose 4B by the cyanogen bromide method.

Equal volumes of settled Sepharose and water were mixed and 200 mg of cyanogen bromide per ml of settled Sepharose was dissolved in the suspension. The reaction temperature was kept at 20 - 25°C and the pH maintained at 11.0. The reaction was completed in 15 minutes. The suspension was filtered and washed quickly with 0.1 M carbonate buffer pH = 8.4 on a Buchner funnel.

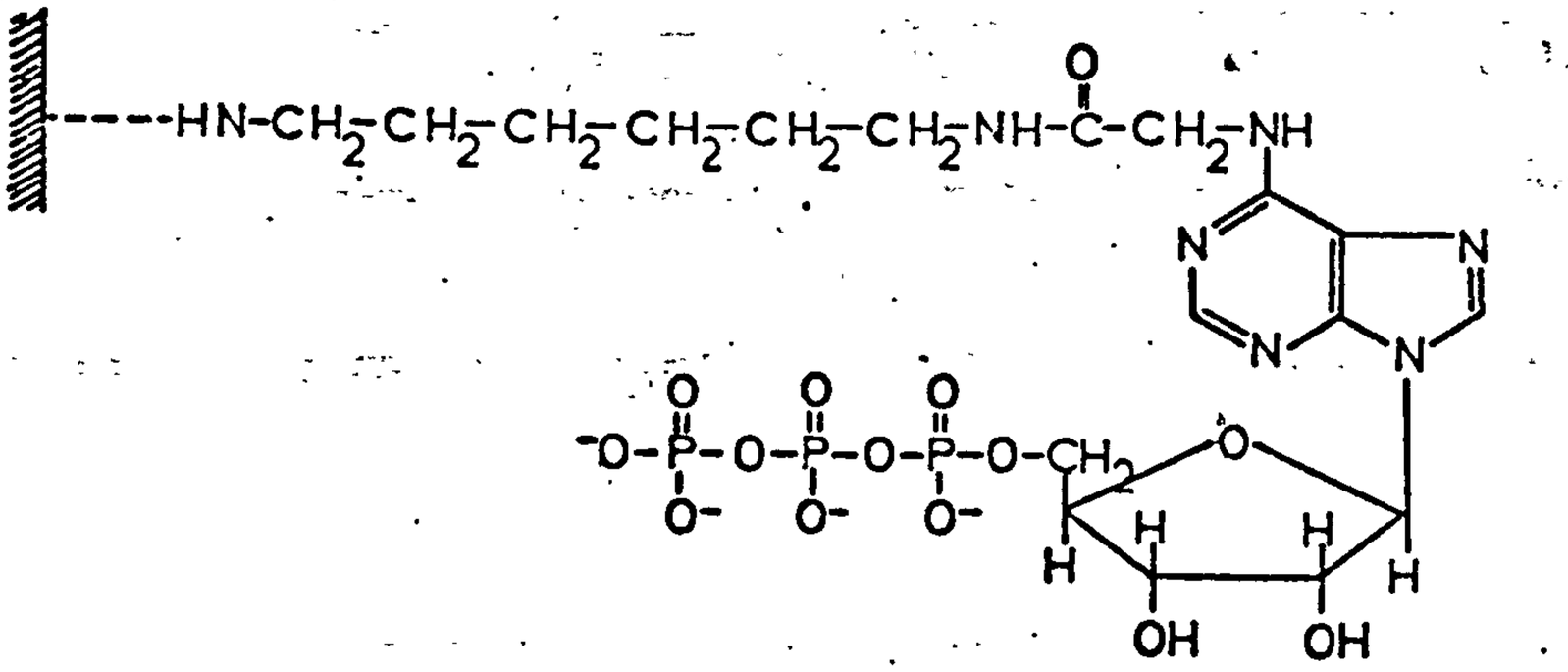


Figure 2.2. N^6-[(6-aminohexyl)carbamoylmethyl]-ATP coupled on Sepharose 4B

(ii) Coupling of N^6-[(6-aminohexyl)carbamoylmethyl]-ATP to the activated Sepharose 4B.

The activated resin was immediately mixed with 1.5 volume of the carbonate buffer containing 5 µmoles of the ATP-analogue per ml of settled Sepharose 4B. The suspension was left for 24 hours under continuous stirring at 4^oC. It was filtered and washed successively with 20 volumes of the following solvents: water, 1 M NaCl, acetate buffer pH = 4.5, I = 0.1 and water. The resin was mixed with 5 volumes of 0.1 M glycine and the suspension was adjusted to pH = 9.5 with 1 N NaOH and stirred overnight at 4^oC for the neutralization of still uncoupled carboxyl groups on the resin. It was further washed with 20 volumes of water, 1 M NaCl and the appropriate buffer for chromatography. Half the quantity of the ATP analogue at the most must have reacted as seen by the absorbance at 267 nm of the reaction medium before and after coupling.

(d) Enzyme assay

Hexokinase was assayed in a Perkin-Elmer 124 spectrophotometer equipped with a 65 chart recorder, fitted with "back off" control. Assays were conducted at 25^oC using a temperature controlled cell compartment. The hexokinase activity was measured by coupling the formation of glucose

6-phosphate to the reduction of NADP to NADPH in the presence of glucose 6-phosphate dehydrogenase. It was assumed that in the presence of NADP all the glucose 6-phosphate was converted to 6-phosphogluconate. The formation of NADPH was followed by continuous recording of the absorbance at 340 nm assuming that the molar extinction coefficient at this wavelength for NADPH is $6.22 \times 10^{-3}\ M^{-1}cm^{-1}$ (Horecker and Kornberg, 1948).

For the measurements of hexokinase activity during the purifications, the assay solution was always freshly prepared and contained:

130 mM TES - KOH buffer pH = 7.2 (I = 0.05)

75 mM KCl -

5 mM glucose

10 mM $MgCl_2$

5 mM ATP

0.6 mM NADP

0.25 $\frac{\text{Units}}{\text{ml}}$ G6PD

Cuvettes of 3 ml capacity and 1 cm light path were used. The reaction was initiated by the addition of 10 - 20 µl of the appropriate dilution of the enzyme solution so that the reaction velocity was linear for at least 10 minutes. In all assays a blank cuvette was inserted in the double beam spectrophotometer containing all the assay

components except hexokinase, thus any hexokinase activity present as impurity in glucose 6-phosphate dehydrogenase was automatically deducted.

(e) Units of enzyme activity

A unit of enzyme activity was defined as the amount of enzyme required to phosphorylate 1 µmole of glucose to glucose 6-phosphate per minute at 25°C, under the assay conditions routinely used. This corresponds to 1 µmole NADPH produced in the coupled assay. Specific activity was defined as units per mg of protein.

(f) Determination of protein concentration

The protein concentration of partially purified samples of hexokinase was calculated using the method of Warburg and Christian (1941), from the absorbance measurements at 260 nm and 280 nm in a 1 cm light path cuvette.

$$\text{Protein concentration (mg/ml)} = 1.55 \times O.D_{280\ nm} - 0.76 \times O.D_{260\ nm}$$

Absorbancies were measured with a Perkin-Elmer 124 spectrophotometer, using 1 cm light path quartz cells, 1.3 ml capacity. This method assumes that a 1 mg/ml protein solution has unit optical density at 280 nm and corrects for any contaminating nucleotides.

The protein concentration of haemolysate was assumed as 35 gr per 100 ml packed erythrocytes.

The protein concentration of the purified heart enzyme was estimated from synthetic boundary measurements in the ultracentrifuge using interference optics (Babul and Stellwagen, 1969). Assuming a constant refractive index for proteins, this method, when compared with the absorbance of the enzyme solution at 280 nm, gave an extinction coefficient, $\varepsilon_{280\ nm}$ for a 1 mg/ml solution of 0.6 (Chapter 9). This value was used only for the estimation of protein concentration in the final purification step of heart hexokinase.

(g) Concentration of dilute protein solutions

At various stages during the purification procedures it was necessary to concentrate protein solutions. Two techniques were used for this purpose.

1. Vacuum dialysis

For small volumes of protein solution, vacuum dialysis was used for concentration purposes. A glass reservoir of 20 - 100 ml capacity was filled with a length of $^{8}/_{32}$" Visking tubing, held in place by a rubber bung. The reservoir and tubing were filled with the dilute protein sol-

ution and placed in a Buchner flask containing buffer to provide concomitant dialysis. The flask was evacuated on a water pump and allowed to stand at $4^{o}C$ with simultaneous stirring of the buffer.

2. Ammonium Sulphate precipitation

For large volumes of protein solution it was found to be most convenient to add sufficient solid enzyme grade ammonium sulphate to precipitate the enzyme and other proteins precipitating at the same salt concentration. Thus concentration and some fractionation was achieved at the same time.

(h) Columns

The columns used for chromatographic experiments were made from borosilicate glass (Pyrex). At their lower end they became narrower and were connected with polythene tubing. The resin was retained in the column by a small quantity of boiled cotton placed at the lower end of the column. The flow of buffer was under gravity, the reservoirs being 10 - 20 cm over the top surface of the resin. Since the flow rate for Sepharose 4B under gravity was very fast, a peristaltic pump was used for slowing down the flow.

CHAPTER 3

Erythrocyte hexokinase.

(a) Introduction

Human erythrocytes have certain advantages for the purification of hexokinase. They represent virtually a single cell population without intracellular organelles and they are easily separated from other components of blood. The problems with lipid and particulate matter are minimal during purification. The ionic and metabolite levels within the erythrocyte have been studied in detail. Haemoglobin constituting approximately 99% of the cell protein can be easily removed in one step (Cohen and Rosemeyer 1969a). The erythrocytes are easily obtained in big quantities from out-dated transfusion blood. Complications arising from the presence of soluble and particulate bound hexokinase are absent since intracellular organelles are missing and only a small amount of hexokinase (6%) has been found associated with the red cell ghosts (Tillmann et al, 1975).

However, in contrast to the importance of hexokinase in the metabolism of human erythrocyte, as a key glycolytic

enzyme in a rather simple and well defined human cell, extensive physicochemical study was hindered by the inability to isolate the enzyme in sufficient quantity. The best previous attempt for purifying a mammalian erythrocyte hexokinase was that of Rijksen and Staal (1976a) from human erythrocytes that led to rather heterogeneous material with a yield of 0.37 mg and a specific activity of 14 Units/mg at 37°C. One of the major difficulties seems to be the level of hexokinase activity in erythrocytes which is the lowest compared with the rest of the glycolytic enzymes (Newsholme and Start, 1973; Chapman et al. 1962; Rapoport, 1968).

The initial aim of this project was to develop a purification procedure for hexokinase from human erythrocytes that would yield sufficient quantities of homogeneous material in order to undertake physical and catalytic studies of the enzyme. In order to overcome the limitation of low hexokinase activity level, the present research was focussed on a big scale purification procedure separating proteins on various principles with the best possible efficiency. This line of investigation unfortunately did not give a homogeneous hexokinase preparation but offered a substantial improvement over previous reports both in yield and purity.

(b) Purification procedure

The scheme that was developed for the purification of hexokinase from human erythrocytes is shown diagrammatically in Figure 3.1. A summary of the purification is given in Table 3.1. Haemolysis and the bulk ion-exchange steps were carried out at room temperature while all other steps were performed at $4^{o}C$.

Step 1. Preparation of haemolysate.

80 pints of blood was centrifuged in an MSE Mistral-6L centrifuge at $4^{o}C$ and 2,000 x g for 10 min. The supernatant plasma and buffy coat were removed by suction. Red cells were washed three times with equal volumes of 0.91% $(\frac{w}{v})$ NaCl + 1 mM K_2EDTA in water and centrifuged as previously. The cells were lysed by mild shaking with equal volumes of water plus 5% $(\frac{v}{v})$ sulphur-free toluene in 10 l glass bottles. Lysis was complete after 20 minutes. The lysate was centrifuged at 2,000 x g for 20 minutes and the upper toluene layer, containing most of the cell debris, was sucked off.

Step 2. Bulk separation on DEAE - Sephadex A-50.

Half of the above haemolysate was diluted with water reducing the ionic strength from 0.15 in the erythrocyte to 0.05. The pH of this solution was titrated to 6.5 with

Figure 3.1. Purification scheme for hexokinase from red cells

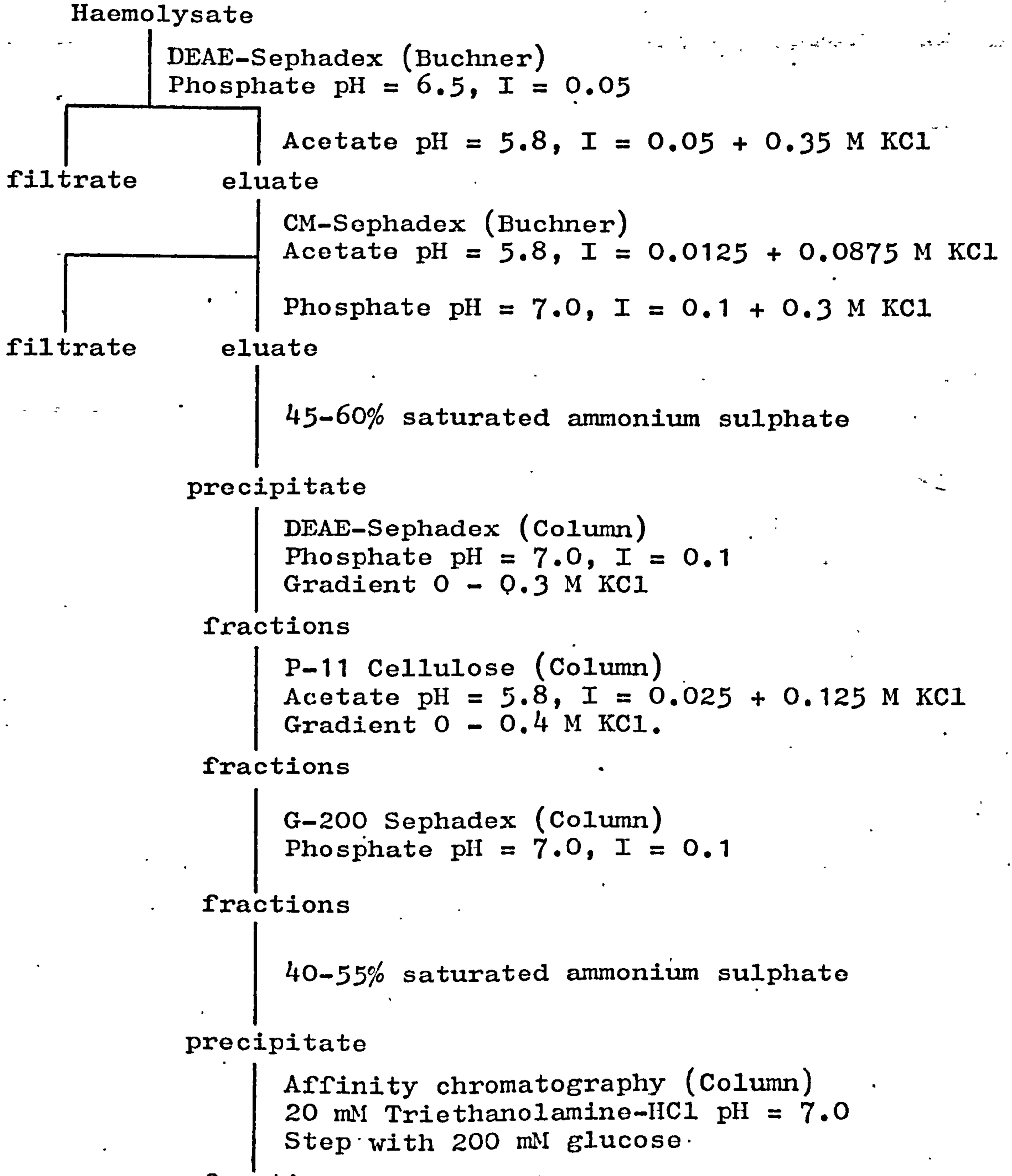


Table 3.1. Summary of a typical purification procedure for hexokinase from erythrocytes

Step	Volume	Protein	Activity	Specific Activity	Purification	Yield
	ml	mg	Units	units/mg	-fold	%
1. Haemolysate	23,000	4.0×10^6	860	0.0002	1	100
2. DEAE-Sephadex*	14,000	-	770	-	-	90
3. CM-Sephadex	8,000	11×10^3	580	0.052	250	68
4. Ammonium sulphate 45 - 60% std.	80	2.3×10^3	336	0.15	714	39
5. DEAE-Sephadex Column	130	580	355	0.61	2,900	41
6. P-11 cellulose	215	150	270	1.8	8,500	31
7. G-200 Sephadex	97	69	250	3.6	17,300	29
8. Ammonium sulphate 40 - 55% std.	2.7	46	165	3.6	17,000	19
9. Affinity chromatography	285	2.9	106	37.0	176,000	12

* Protein concentration was not measured at Step 2 because of the high absorbance at 260 nm

0.5 M K_2HPO_4. The diluted haemolysate was pumped equally into two 25 l aspirators and allowed to flow through two 24 cm (i.d.) Buchner funnels, each containing 2 lt of volume of DEAE-Sephadex equilibrated with phosphate buffer pH = 6.5, I = 0.05. A constant head of liquid was maintained on the funnel by a level control (Pearse, Ph.D. Thesis, 1972) and the filtrate was syphoned away without disturbing the funnel and the resin. The flow rate through each Buchner funnel was about 7 lt per hour. Two thicknesses of Whatman 54 filter paper were used to retain the resin and one was placed on the surface as an anti-disturbance disc. The resin in each funnel was washed with phosphate buffer pH = 6.5, I = 0.05 until the filtrate was colourless, applying mild suction after the last wash. The enzyme was eluted with acetate buffer pH = 5.8, I = 0.05 + 0.35 M KCl, using 2.5 lt of buffer for each Buchner funnel, the resin being stirred and sucked dry during the final stages of the elution. The eluate was made 2 mM in 6-aminohexanoic acid since it is thought to act as inhibitor of the proteolytic enzyme plasmin (Yoshida, 1966). The solution was stored at $4^{o}C$, while step 2 was repeated during the next day on the second half of the above haemolysate. A total of 300 g of DEAE-Sephadex was used.

Step 3. Bulk separation on CM - Sephadex C-50.

The combined eluates from the DEAE-Sephadex were diluted with water to decrease the ionic strength to 0.1. The solution was titrated to pH=5.8 with 10% acetic acid. It was applied as before, to two 24 cm Buchner funnels each containing a 2.5 l bed volume of CM-Sephadex, equilibrated with acetate buffer pH=5.8, I=0.0125 + 0.0875M KCl. The flow rate was about 7 l per hour. The resin was washed with 10 lt of the above buffer and eluted with 3 l of phosphate buffer pH=7.0, I=0.1 + 0.3M KCl. The resin was stirred and sucked dry in the final stage of elution. A total of 150 g of CM-Sephadex was used.

Step 4. First fractionation with ammonium sulphate.

To each l of eluate 277 g of ammonium sulphate were added gradually with stirring to give a 45% saturation with the salt. The precipitate was allowed to form on overnight storage at 4^{o}C. It was centrifuged at 10,000 x g for 20 min using a Sorvall RC2-B preparative centrifuge. The precipitate was discarded and the supernatant was made 60% saturated in ammonium sulphate by addition of 99 g of the salt per l. The precipitate was allowed to form on storage as before. The suspension was centrifuged as above. The supernatant was decanted and the precipitate was dissolved in a small volume of phosphate buffer pH=7.0, I = 0.1 and dialysed against 60 volumes of the same buffer. The dialysis buffer was replaced twice.

Step 5. Chromatography on DEAE - Sephadex A-50

Any precipitate remaining after dialysis was removed by centrifugation at 15,000 x g for 10 minutes. The solution was applied to a 30 x 2.5 cm column equilibrated with phosphate buffer pH = 7.0, I = 0.1. A linear salt gradient was applied using two identical interconnected 250 ml vessels, one containing the above phosphate buffer; the other containing the phosphate buffer with 0.3 M KCl added. The flow rate was 50 ml/hour and 4 ml fractions were collected. The elution profile is given in Figure 3.2. Fractions were pooled as shown and dialysed against 25 volumes of acetate buffer pH = 5.8, I = 0.025 + 0.125 M KCl with three changes of buffer.

Step 6. Chromatography on P-11 cellulose.

Any precipitate remaining after dialysis was removed by centrifugation at 15,000 x g for 10 minutes. The solution was applied to a 30 x 2.5 cm column. The resin was equilibrated with acetate buffer pH = 5.8, I = 0.025 + 0.125 M KCl. A linear salt gradient was applied using two identical interconnected 250 ml vessels, one containing the above buffer and the other the same buffer plus 0.4 M KCl. The flow rate was 70 ml/hour and 4.5 ml fractions were collected. The elution profile is given in Figure 3.3. Fractions were pooled as indicated and were concentrated to

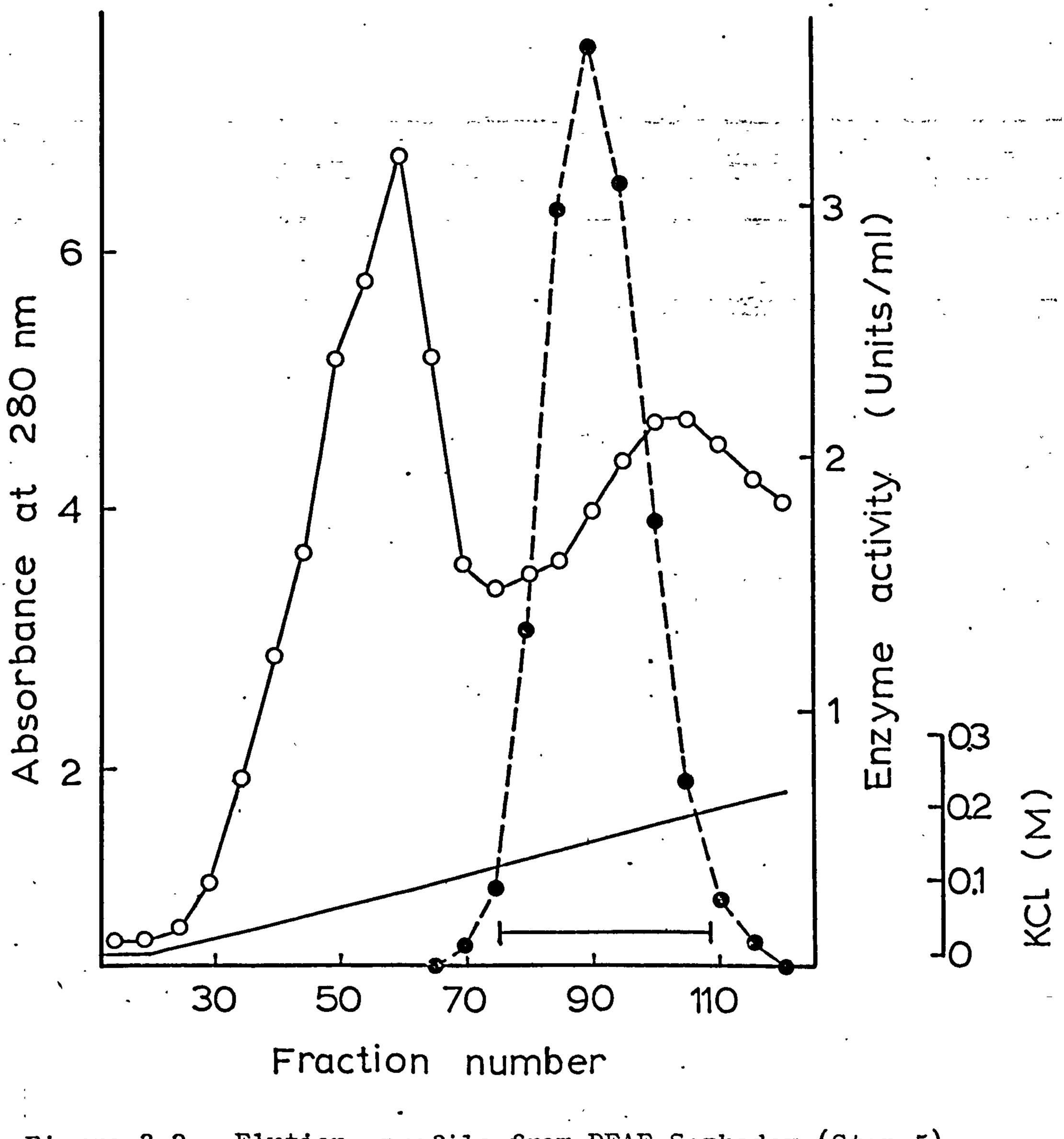


Figure 3.2. Elution profile from DEAE-Sephadex (Step 5)

(○—○) Absorbance at 280 nm; (●--●) Enzyme activity. Fractions of 4 ml were collected. The horizontal line indicates the fractions pooled after elution.

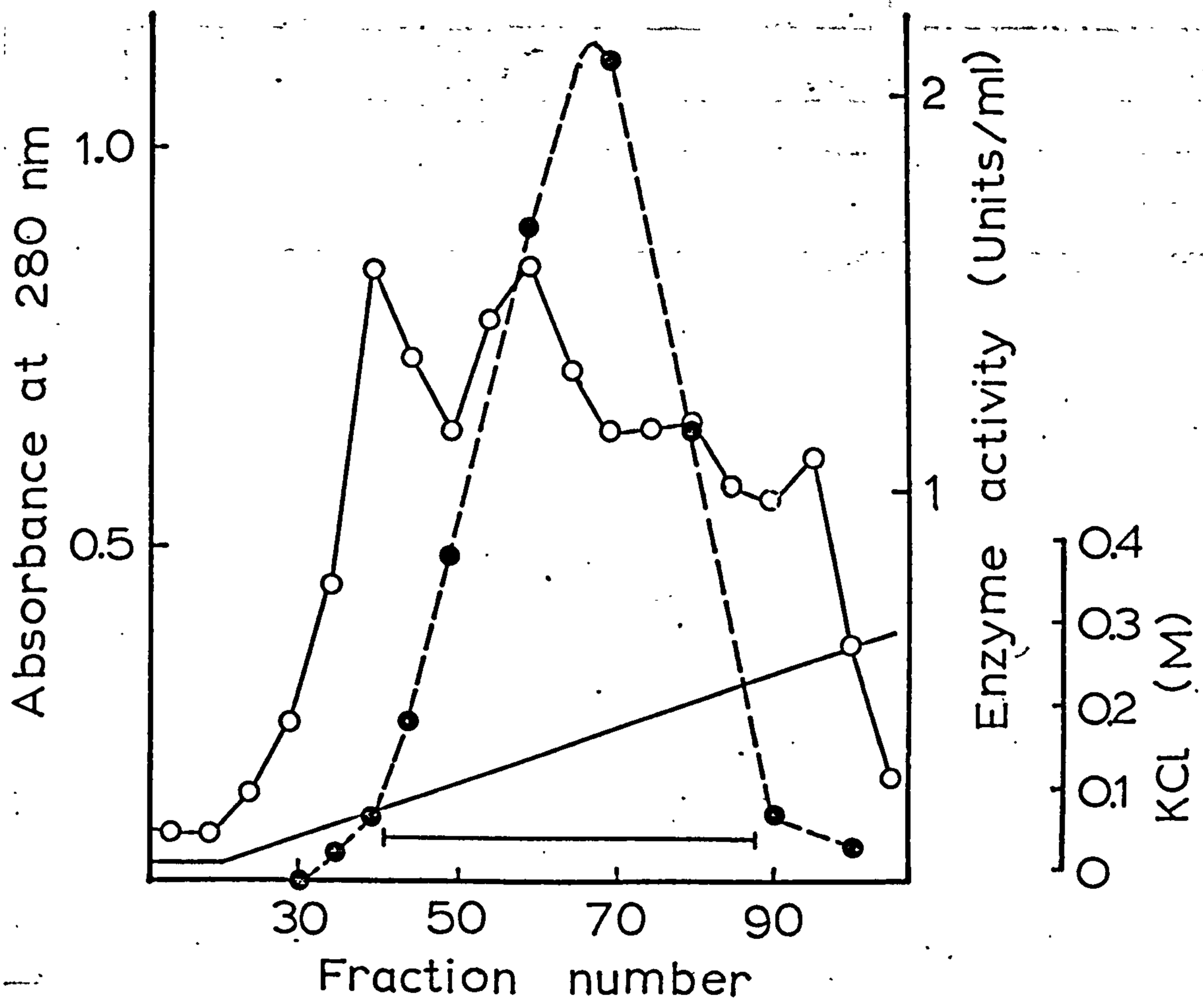


Figure 3.3. Elution profile from P-11 cellulose (Step 6)

(O—O) Absorbance at 280 nm; (●--●) Enzyme activity.

Fractions of 4.5 ml were collected. The horizontal line indicates the fractions pooled after elution.

2 ml with vacuum dialysis against phosphate buffer pH = 7.0, I = 0.1.

Step 7. Gel filtration on Sephadex G-200.

The dialysed solution was centrifuged at 15,000 x g for 10 minutes to remove any precipitate and was applied to a 150 x 2 cm column of Sephadex G-200, which had been equilibrated with phosphate buffer pH = 7.0, I = 0.1. The same buffer was used for elution. The flow rate was about 7 ml/hour and 4 ml fractions were collected. The elution profile is shown in Figure 3.4. Fractions were pooled as indicated and concentrated to 3 ml with vacuum dialysis.

Step 8. Second fractionation with ammonium sulphate.

The concentrated protein solution was centrifuged on a bench centrifuge for 10 minutes to remove any precipitate. A saturated solution of ammonium sulphate was added dropwise with stirring, to give a faint turbidity which occurred at about 40% saturation with ammonium sulphate. After leaving the suspension for 3 hours at room temperature, it was centrifuged as above and the precipitate was discarded. The supernatant was made 55% saturated in ammonium sulphate in the same way as before and left for 3 hours at room temperature. After centrifugation the precipitate was dissolved in 4 ml of 20 mM Triethanolamine

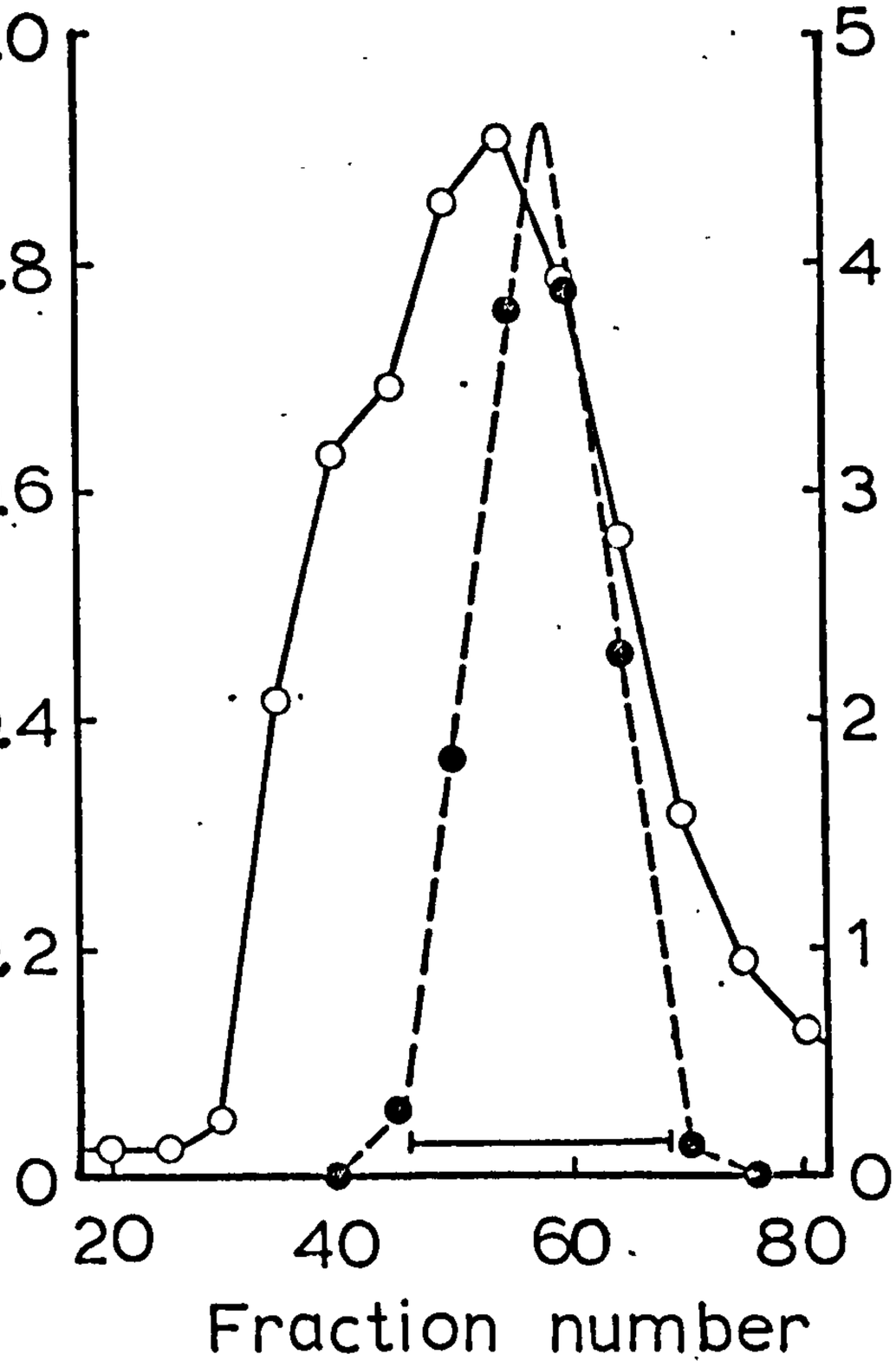


Figure 3.4. Elution profile from gel-filtration on Sephadex G-200 (Step 7)

(○—○) Absorbance at 280 nm; (●--●) Enzyme activity. Fractions of 4 ml were collected. The horizontal line indicates the fractions pooled after elution.

buffer pH = 7.0 and dialysed against 1,000 volumes of the same buffer without glucose and with one change.

Step 9. Affinity chromatography.

After centrifugation at 15,000 x g for 10 minutes, the sample was applied to a column 18 x 2.8 cm of Sepharose 4B on which glucosamine was bound with the aid of 6-amino-hexanoic acid acting as spacer. The resin was equilibrated with 20 mM Triethanolamine-HCl buffer pH = 7.0 without glucose. A volume of 125 ml of the same buffer was passed through the column to remove proteins not bound on the resin. With the subsequent passage of 240 ml of the same buffer plus 50 mM KCl, a smaller protein peak was eluted. The enzyme was eluted specifically with buffer containing 200 mM glucose. The flow rate was adjusted to 35 ml/hour with a peristaltic pump and fractions of 5.2 ml were collected. The elution profile is given in Figure 3.5. The protein content of the fractions with hexokinase activity was very low and could not be detected by measuring absorbance at 280 nm. Fractions were pooled as indicated and concentrated to a small volume, with protein concentration of 4 mg/ml, by vacuum dialysis against phosphate buffer pH = 7.0, I = 0.1. The hexokinase preparation was stored at $4^{o}C$ under nitrogen.

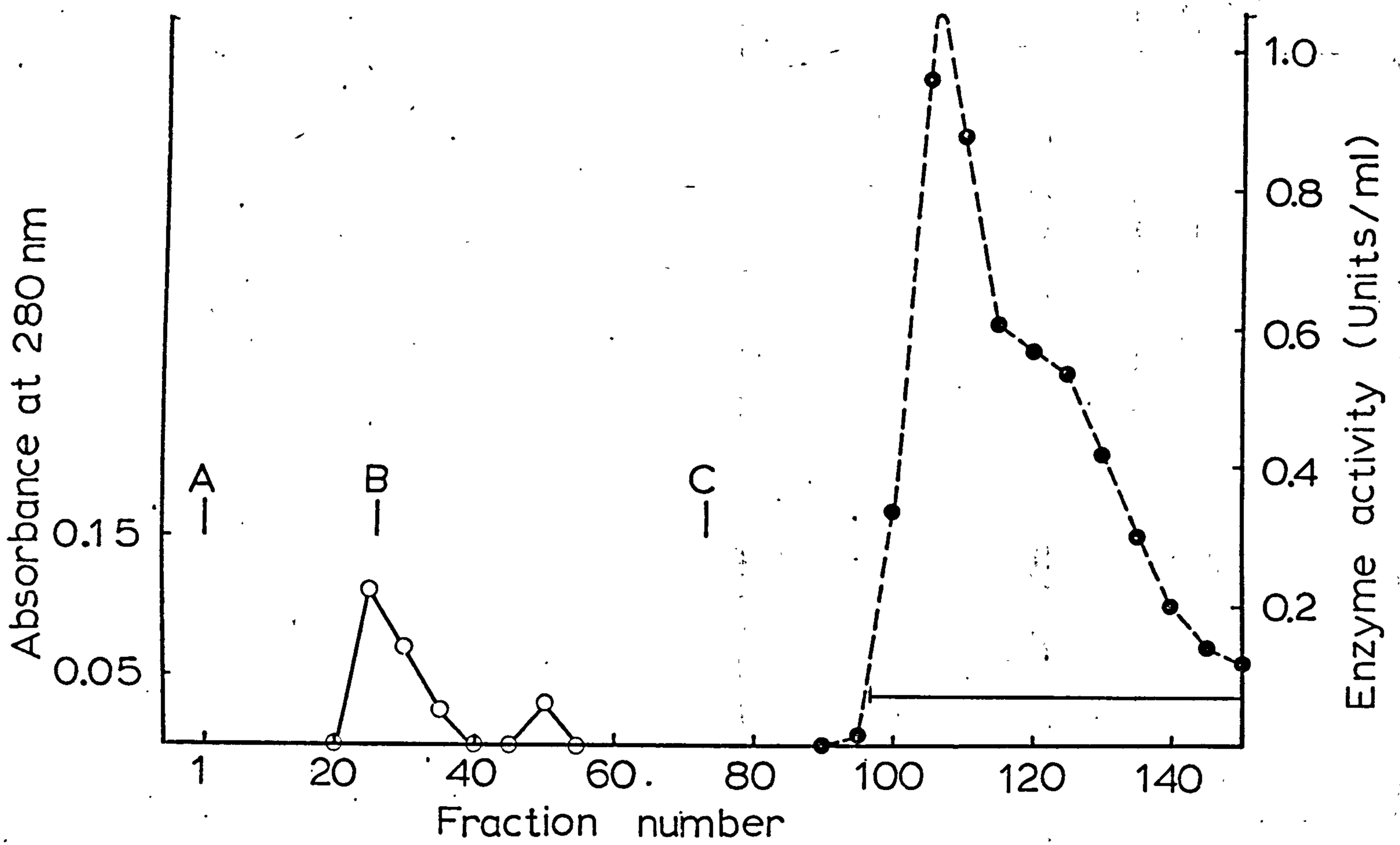


Figure 3.5. Elution profile from the affinity chromatography (Step 9)

(O—O) Absorbance at 280 nm; (●—●) Enzyme activity. Fractions of 4 ml were collected. The horizontal line indicates the fractions pooled after elution.

A: 20 mM Triethanolamine-HCl buffer pH = 7.0; B: Buffer A containing 50 mM KCl; C: Buffer B containing 200 mM glucose. The trail in the activity peak was caused by interrupting the flow overnight.

(c) Comments on the purification procedure

The cells haemolised represented mainly erythrocytes since plasma was removed by successive washings of the packed red cells with physiological saline and leucocytes were removed by sucking off the buffy coat and supernatant after centrifugation of blood.

In the body, erythrocytes have a finite life span of approximately 120 days after which they are removed from circulation by the reticuloendothelial system. So erythrocytes in blood represent all ages from 0 to 120 days. In the out of date transfusion blood used in this purification the youngest cells are 21 days old. It has been shown that the activity of some enzymes including hexokinase deteriorated with senescence, the half-life time for hexokinase being 21 days (Chapman and Schaumberg, 1967). The level of this enzyme in mature erythrocytes represented an estimated 2-3% (Rogers et al, 1975a) or a measured 14% (Chapman and Schaumberg, 1967) of the reticulocyte activity (young erythrocytes, 0-2 days old containing reticulum and thus having the potential to synthesize some proteins). These facts explain the low specific activity for hexokinase found in haemolysates of this purification e.g. 0.0002 units/mg at 25°C. This is near the values found in haemolysates by other investigators e.g. 0.00042 units/mg (assay at 37°C) by Gerber et al., 1974 and 0.00047 units/mg

(assay at 37°C) by Rijksen and Staal, 1976a.

The following experiment suggested that significant amounts of particulate hexokinase were not present. Equal volumes of packed erythrocytes and phosphate buffer pH=7.0, I = 0.02 were mixed and haemolysis was complete in 30 minutes at room temperature with mild agitation. The haemolysate was centrifuged for 20 minutes at 27,000 x g and the pellet was divided and suspended with one volume of water and one volume of acetate buffer pH = 5.6, I = 0.1 containing 0.2 M $MgCl_2$ respectively. After 30 minutes incubation at 4°C the suspensions were centrifuged as above. With both extraction buffers, the enzyme activity found in the supernatant represented 3-4% of that found in the supernatant of haemolysate. A second extraction of the pellet, as described above, did not yield any enzyme activity. It seems that there is no firmly bound particulate hexokinase and that the small activity found in the first extraction represented hexokinase entrapped in the pellet.

The procedure developed for the purification of erythrocyte hexokinase makes use of differences between the enzyme and other erythrocyte proteins on such properties as positive and negative charges, solubility in presence of salt, molecular size and specificity for immobilized glucosamine.

The initial DEAE-Sephadex step was designed for the removal of haemoglobin that constituted the bulk of protein (4 Kg) present in the haemolysate. Although protein measurements were not made after the DEAE-Sephadex step because of the high readings at 260 nm, only 11g were left after the subsequent CM-Sephadex (Buchner) step.

Both the initial DEAE and CM-Sephadex steps were performed as stepwise arrangements on Buchner funnels for the faster removal of the bulk of protein and for ease of handling large volumes of haemolysate and DEAE-Sephadex eluate. These volumes would be difficult to dialyse and so the reduction in ionic strength was achieved by dilution. Effective resolution on the resins was obtained by later steps in the preparation.

In the presence of 10 mM glucose, 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA, 25% of enzyme activity was lost on the CM-Sephadex step. If glucose and 2-mercaptoethanol were not incorporated in the buffers the loss was sharply increased to 70%. In contrast to this, the absence of glucose and 2-mercaptoethanol had no effect in the recovery from the initial DEAE-Sephadex step (90%). Glucose is known to stabilize mammalian hexokinases against inactivation by heat and proteolysis (Grossbard and Schimke, 1966; Wilson, 1978) and sulfhydryl reagents (Redkar and Kenkare, 1975; Chou and Wilson, 1974).

The fractionation with ammonium sulphate (Step 4) concentrated the protein solution for subsequent chromatography and gave a 2.5 fold purification.

Hexokinase behaved as a single peak in both anion (Step 5) and cation (Step 6) column chromatographic steps. On Whatman P-11 cellulose the hexokinase activity peak was rather broad. Before deciding on Whatman P-11 cellulose as the best cation exchanger this resin was compared with CM-Sephadex C-50 and CM Bio-Gel A. All were equilibrated with acetate buffer pH = 5.8, I = 0.01 containing 0.05 M KCl. Hexokinase was eluted with a 500 ml linear salt gradient of 0-0.4 M KCl. In all three experiments a single enzyme peak was obtained but the specific activities of the recovered hexokinase were 1/3 for the CM-Sephadex C-50 column and 2/3 for the CM Bio-Gel A column of the enzyme's specific activity from the Whatman P-11 cellulose column. The recoveries were similar. One peak of activity was also obtained with the anion exchange DEAE Bio-Gel A with the same recovery and specific activity as with the hexokinase from the DEAE-Sephadex A-50 column.

The effect of the second fractionation with ammonium sulphate (Step 8) on purification ranged from a factor of 1 to 1.5 and could be omitted.

Sephadex G-200 gel filtration and the affinity chromatography were left to the end of the purification

procedure for best effect i.e. enhanced resolution in gel-filtration and less non-specific binding on affinity chromatography. The gel filtration revealed size heterogeneity with protein species mainly of bigger size than that of hexokinase. The affinity step was the most effective, offering a ten-fold purification and thus raising the purification factor from 17,000 to 170,000.

(d) Conclusions

The purification procedure developed for human erythrocyte hexokinase contains 9 steps and lasts for less than three weeks. Beginning with 80 pints of blood, 3 mg of protein was recovered with a specific activity of 37 units/mg. The overall yield was rather low (12%) and the overall purification high (170,000).

From experiments reported in Chapter 8, it is obvious that the material is still impure. From evidence as the specific activity and SDS electrophoresis it seems that a 2 to 3 fold purification is still needed for the isolation of the enzyme. Since the quantity of purified protein was small and the obvious purification techniques were exhausted, no attempt for further purification of the enzyme was made.

Two previous purifications of mammalian erythrocyte hexokinase are reported, both for the human enzyme (Table

3.2). An improvement was made over the best of them (Rijksen and Staal, 1976a) for the following reasons. The procedure was scaled up for dealing from approximately 4 pints of blood to 80 by solving technical problems involved in big scale laboratory preparations. Cation exchange steps were added for the removal of some negatively charged proteins. These factors resulted in 3 mg of protein with a specific activity of 37 units/mg (25°C) compared with 0.4 mg of protein with specific activity of 14 units/mg (37°C).

This preparation suggests that the low hexokinase activity in haemolysates is due to low levels of protein rather than a low specific activity. Further purification would be required for sufficient material to study the physical properties of erythrocyte hexokinase. Such attempts would demand larger amounts of starting material and some improvement in the recoveries and resolutions for the purification steps.

Table 3.2. Partial purification of human erythrocyte hexokinase

Reference	Specific activity	No of steps	Yield	Purified protein	Protein measurement	Assay solution	Temperature
	units/mg		%	mg			°C
Gerber et al. (1974)	0.29	6	20.5	101	-	75 mM TES pH = 7.2, 5 mM ATP, 10 mM $MgCl_2$, 5 mM glucose, 0.25 units/ml G6PD, 0.6 mM NADP, 75 mM KCl.	37
Rijksen and Staal (1976a)	14.3	6	14	0.37	Lowry	33 mM Tris-HCl pH = 7.25 5 mM ATP, 10 mM $MgCl_2$, 10 mM glucose, 0.05 units/ml G6PD, 0.33 mM NADP.	37
Present results	37	9	12	2.9	$A^{1\%}_{280nm}=10$	130 mM TES-KOH, pH = 7.2 5 mM ATP, 10 mM $MgCl_2$, 5 mM glucose, 0.25 units/ml G6PD, 0.6 mM NADP, 7.5 mM KCl.	25

CHAPTER 4

Placental hexokinase.

(a) Introduction

Placenta was used as a readily obtainable tissue of interest as a source of human foetal material. As no other attempted isolation of hexokinase from such a source has been reported, this preliminary isolation is mentioned although it was only carried out on small-scale pilot experiment.

(b) Purification procedure

The scheme used for the partial purification of hexokinase from human term placenta is shown diagrammatically in Figure 4.1. A summary of the above purification is given in Table 4.1. All steps were carried out at $4^{o}C$.

Step 1. Solubilization of hexokinase

One placenta was left to thaw out at $4^{o}C$. A quantity of 50 gr of tissue was homogenised with two volumes of chilled phosphate buffer pH = 7.0, I = 0.02 in a Waring blender for four 20 second intervals at full speed. The

Figure 4.1. Purification scheme for hexokinase from term placenta

Homogenate

Solubilization
Acetate pH = 5.6, I = 0.1 + 0.2 M $MgCl_2$

solution

Dialysis
Phosphate pH = 8.0, I = 0.1

solution

DEAE-Sephadex (Column)
Phosphate pH = 8.0, I = 0.1
Gradient 0 - 0.4 M KCl

fractions

P-11 cellulose (Column)
Acetate pH = 5.8, I = 0.025 + 0.125 M KCl
Gradient 0 - 0.4 M KCl

fractions

Sephadex G-200 (Column)
Phosphate pH = 7.0, I = 0.1

fractions

Table 4.1. Summary of procedure for purification of hexokinase from term placenta

	Step	Volume	Protein	Activity	Specific Activity	Yield
		ml	mg	Units	Units/mg	%
1.	Solubilization	109	*	39	—	100
2.	Dialysis	109	1.01×10^3	34.5	0.034	89
3.	DEAE-Sephadex	265	167	31.8	0.19	82
4.	P-11 cellulose	111	14.3	21.5	1.5	55
5.	Sephadex G-200	59	1.9	16.1	8.5	41

* The extract was turbid and the initial estimate of protein concentration was unreliable.

homogenate was centrifuged for 20 minutes at 35,000 x g. The supernatant was decanted and the pellet was mixed with 2 volumes of acetate buffer pH = 5.6, I = 0.1 containing 0.2 M $MgCl_2$ and homogenised as before. The suspension was left to stand for 45 minutes and centrifuged as above.

Step 2. Dialysis.

The pellet was discarded and the supernatant was dialysed against 30 volumes of phosphate buffer pH = 8.0, I = 0.1 with 2 changes. A heavy precipitate was formed, that was removed by centrifugation at 10,000 x g for 20 minutes.

Step 3. Chromatography on DEAE-Sephadex A-50.

The supernatant was applied to a 21 x 2.5 cm column equilibrated with phosphate buffer pH=8.0, I=0.1. A linear salt gradient was applied using two identical interconnected 250 ml vessels, one containing the phosphate buffer pH=8.0, I=0.1, the other containing the same buffer plus 0.4 M KCl. The flow rate was 30 ml/hour and 4.5 ml fractions were collected. The elution profile is given in Figure 4.2. Fractions were pooled as shown and were dialysed against 25 volumes of acetate buffer pH=5.8, I=0.025 containing 0.125 M KCl with one change of buffer.

Step 4. Chromatography on P-11 cellulose.

The dialysed solution was centrifuged at 15,000 x g for 10 minutes to remove any precipitate formed during dialysis and was applied to a 17x2.5 cm column equilibrated with acetate buffer pH=5.8, I=0.025+0.125 M KCl. A linear gradient was applied using two identical interconnected 250 ml vessels,

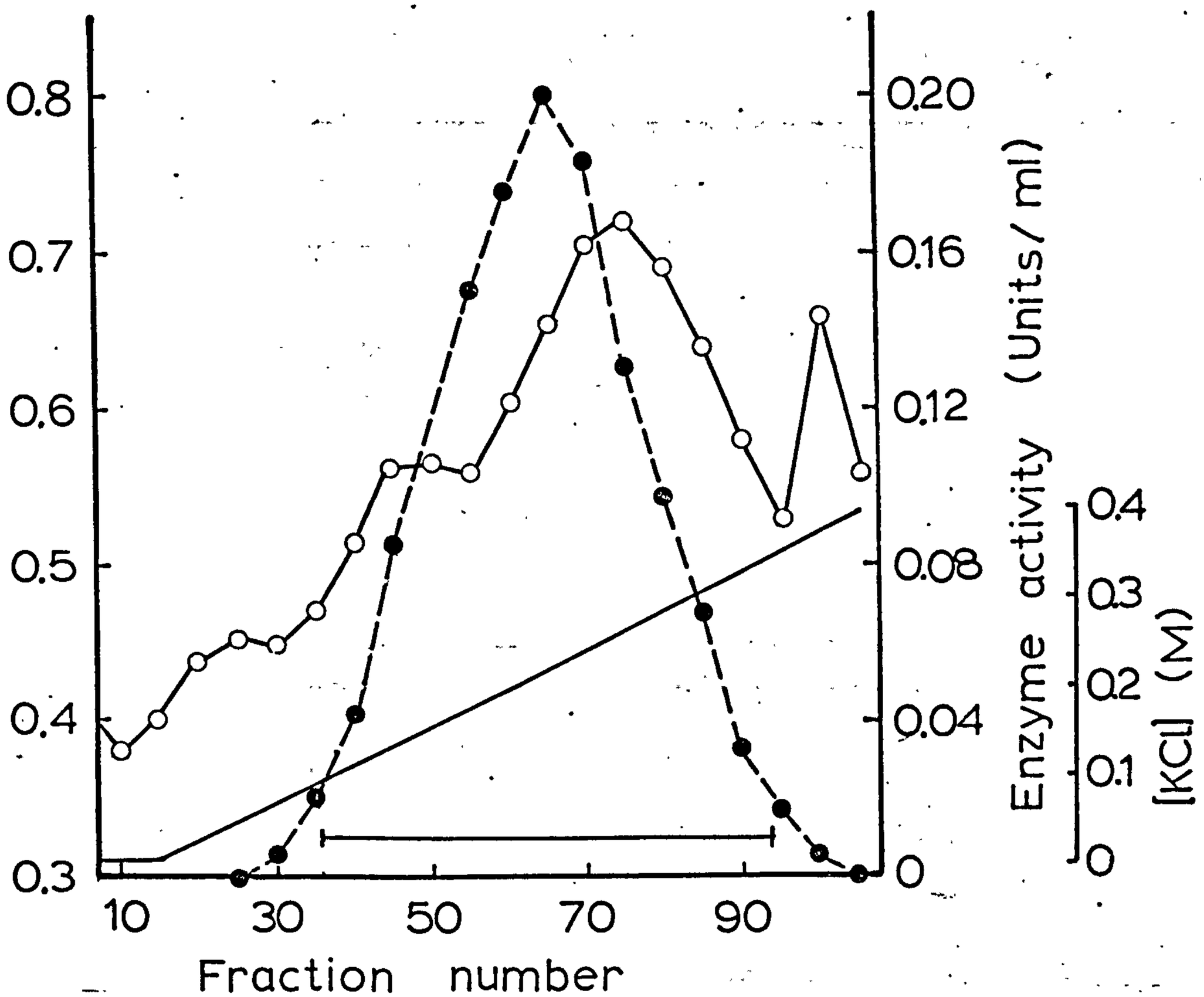


Figure 4.2. Elution profile from DEAE-Sephadex (Step 3)

(O—O) Absorbance at 280 nm; (●—●) Enzyme activity. Fractions of 4.5 ml were collected. The horizontal line indicates the fractions pooled after elution.

one containing the acetate buffer and the other the same buffer plus 0.4 M KCl. The flow rate was 50 ml/hour and 4.8 ml fractions were collected. The elution profile is given in Figure 4.3. Fractions were pooled as shown and were concentrated by vacuum dialysis to 1.6 ml and dialysed against 500 volumes of phosphate buffer pH = 7.0, I = 0.1 with one change of buffer.

Step 5. Gel filtration on Sephadex G-200.

The dialysed solution was centrifuged at 15,000 x g for 10 minutes to remove any precipitate formed during dialysis and was applied to a 150 x 2 cm column of Sephadex G-200 which had been equilibrated with phosphate buffer pH = 7.0, I = 0.1. The same buffer was used for elution. The flow rate was 7 ml/hour and 4 ml fractions were collected. The elution profile is shown in Figure 4.4. Fractions were pooled as indicated and concentrated by vacuum dialysis to a small volume.

(c) Comments on the purification procedure

The extraction of 50 g of placental tissue with phosphate buffer pH = 7.0, I = 0.02 gave 15 units of enzyme activity in the supernatant. Subsequent two extractions with acetate buffer pH = 5.6, I = 0.1 with 0.2 M $MgCl_2$ gave 39 and 12 units of enzyme activity respectively.

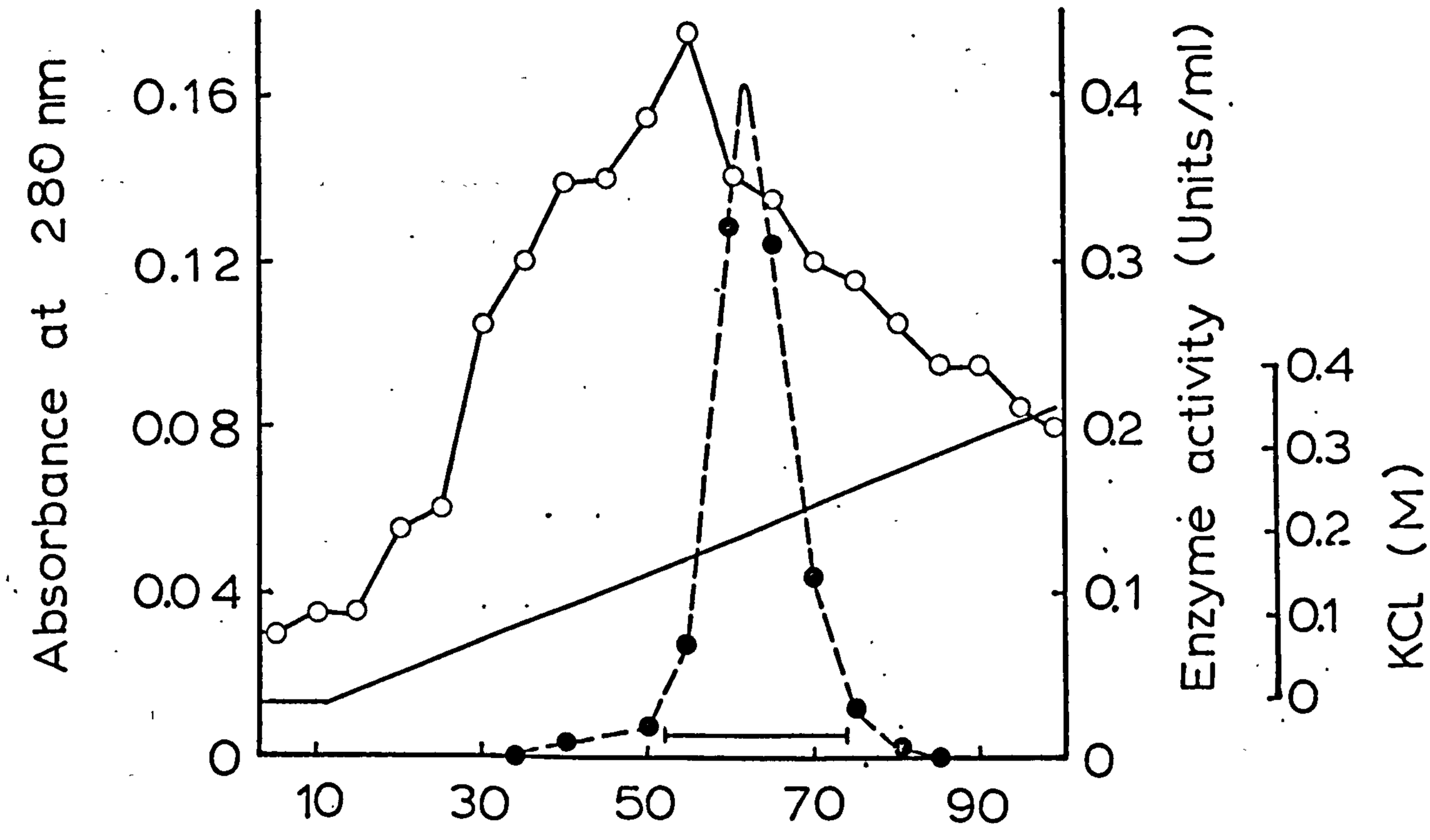


Figure 4.3. Elution profile from P-11 cellulose (Step 4)

(O—O) Absorbance at 280 nm; (●—●) Enzyme activity. Fractions of 4.8 ml were collected. The horizontal line indicates the fractions pooled after elution.

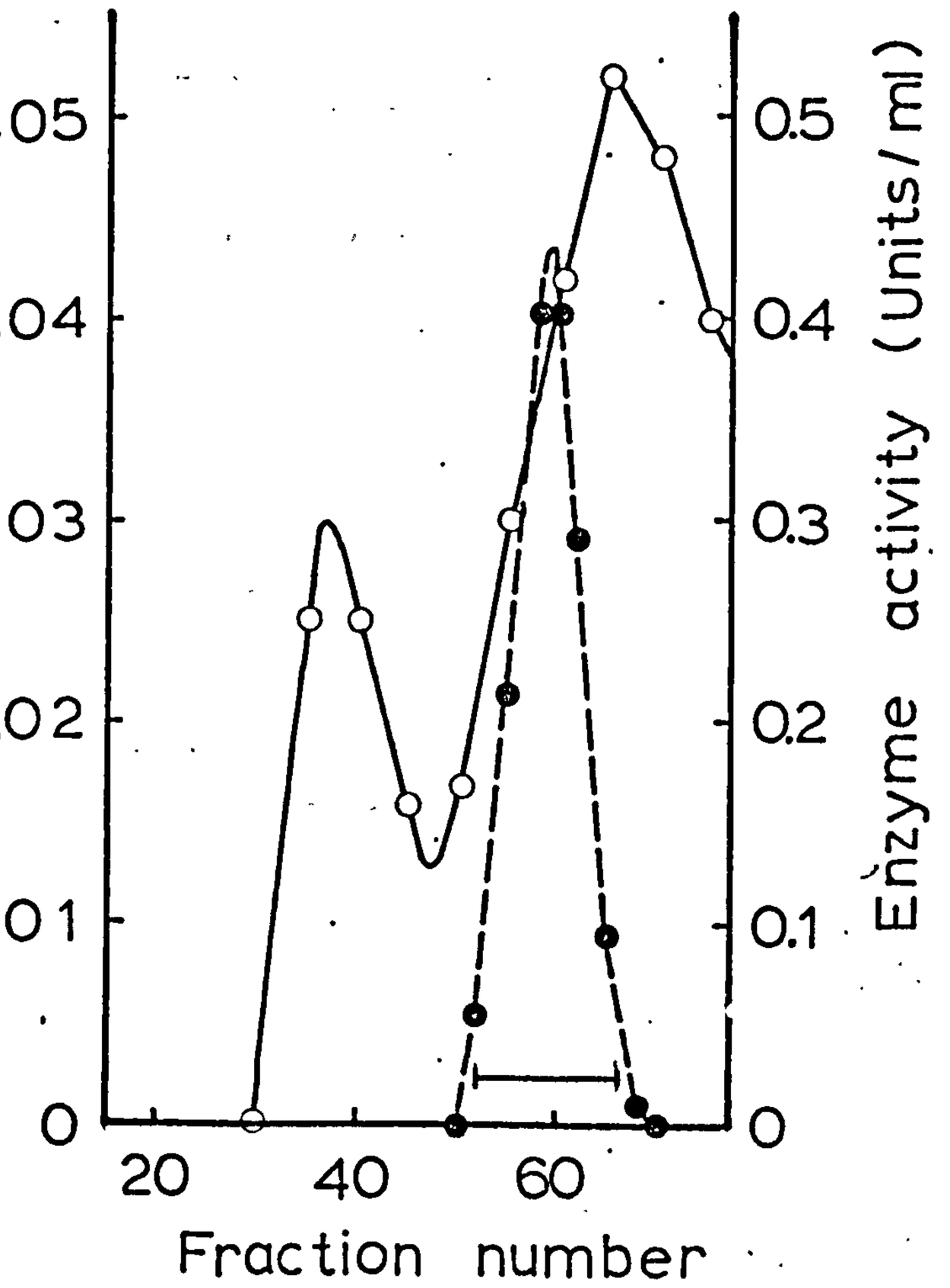


Figure 4.4. Elution profile from gel-filtration on Sephadex G-200 (Step 5)

(O—O) Absorbance at 280 nm; (●—●) Enzyme activity. Fractions of 4 ml were collected. The horizontal line indicates the fractions pooled after elution.

This is an indication that most of the placental hexokinase is of the particulate form and that an increased salt concentration is needed for its extraction. The tissue was not perfused, only drained of blood, but the contribution of erythrocyte hexokinase should be minimal because of its' low specific activity. Placenta under the conditions mentioned is at least six times richer in hexokinase than erythrocytes if the total activity recovered as above is expressed per g fresh tissue and compared with the erythrocyte hexokinase activity expressed per g of haemoglobin. The proportion of hexokinase activity found in the supernatant of homogenate as compared to the sum of enzyme activity extracted with $MgCl_2$ is within the range of 20-30% of hexokinase activity found in the cytoplasmic supernatant of human term placentas by Gustke and Kowalewski (1975). The freezing of the tissue at -25°C and thawing possibly does not increase the soluble activity but exposes latent activity (also released by salts) as was found to be the case for rat brain hexokinase (Wilson, 1967, 1968; Teichgräber and Biesold, 1968).

The purification procedure followed was that used for the purification of porcine heart hexokinase on a large scale by Easterby and O'Brien (1973) with the following minor modifications. The P-11 cellulose resin was equilibrated with acetate buffer pH = 5.8, I = 0.025 containing

0.125 M KCl instead of phosphate buffer pH = 6.5, I = 0.05 and the salt gradient was 0-0.3 M KCl instead of 0-0.4 M KCl. Also the G-200 Sephadex resin was equilibrated with phosphate buffer pH = 7.0, I = 0.1 instead of phosphate buffer pH = 8.0, I = 0.1 and the gradient volume for DEAE-Sephadex chromatography was 500 ml instead of 2 lt.

All three chromatographic steps showed a purification factor of 6 to 8. The low specific activity of the dialysed extract indicate that placenta is a poor tissue for providing pure material, using the above procedure.

(d) Conclusions

Hexokinase was purified at least 200-fold from human placenta with 5 steps in 9 days to a specific activity of 8.5 units/mg. The only previous report for purification of hexokinase from placenta is a 15-fold purification from human term placenta by Gustke (1975).

No further purification of hexokinase was pursued from this tissue since hexokinase from heart purified with the same small scale procedure gave a better final specific activity of 20 units/mg and thus the investigation was transferred to the heart enzyme (Chapter 5).

CHAPTER 5

Heart hexokinase.

(a) Introduction

Cardiac muscle is a tissue richer in hexokinase than erythrocytes. Although it contains less hexokinase than brain, heart has a high percentage of soluble enzyme (Wilson and Felgner, 1977) and it is a tissue that is easily disrupted and has a low lipid content. From previous reports human heart should contain only one isoenzyme, HKI (Neumann et al., 1974; Rogers et al., 1975b).

Easterby and O'Brien (1973) obtained pure enzyme from porcine hearts, in sufficient quantity for physicochemical studies. The only previous purification of hexokinase from human heart did not give material of high purity in sufficient yield (Neumann et al., 1974).

With the purification procedure presented here, 25 mg of well characterized protein was recovered from 1.15 Kg of human heart tissue.

(b) Purification procedure

The scheme that was developed for the purification of hexokinase from human heart is shown diagrammatically

in Figure 5.1. A summary of the above purification is given in Table 5.1. All steps were carried out at $4^{o}C$ except the bulk separation on DEAE-Sephadex.

Step 1. Preparation of homogenate.

Refrigerated hearts were left to thaw at $4^{o}C$. Adjacent fat and connective tissue was removed. The muscle was cut into small pieces and minced. A quantity of 1.15 Kg of minced tissue was homogenised with three volumes of chilled phosphate buffer pH = 7.5, I = 0.02 in a Waring blender for four 20 second intervals in full speed. The homogenate was centrifuged in a Sorvall RC2-B preparative centrifuge for 15 minutes at 6,000 x g. The pellet was extracted once more with three volumes of the same buffer and centrifuged as above. The supernatants of both extractions were pooled and passed through glass wool to remove floating lipid.

Step 2. Bulk separation on DEAE - Sephadex A-50.

The pH of the protein solution was about 6.0 and was titrated to 6.5 with 0.5 M K_2HPO_4. The solution was divided into two halves and allowed to flow under gravity through two 24 cm Buchner funnels, each containing a 2 lt bed volume of DEAE-Sephadex equilibrated with phosphate buffer pH = 6.5, I = 0.05. The flow rate through each

Figure 5.1. Purification scheme for hexokinase from heart

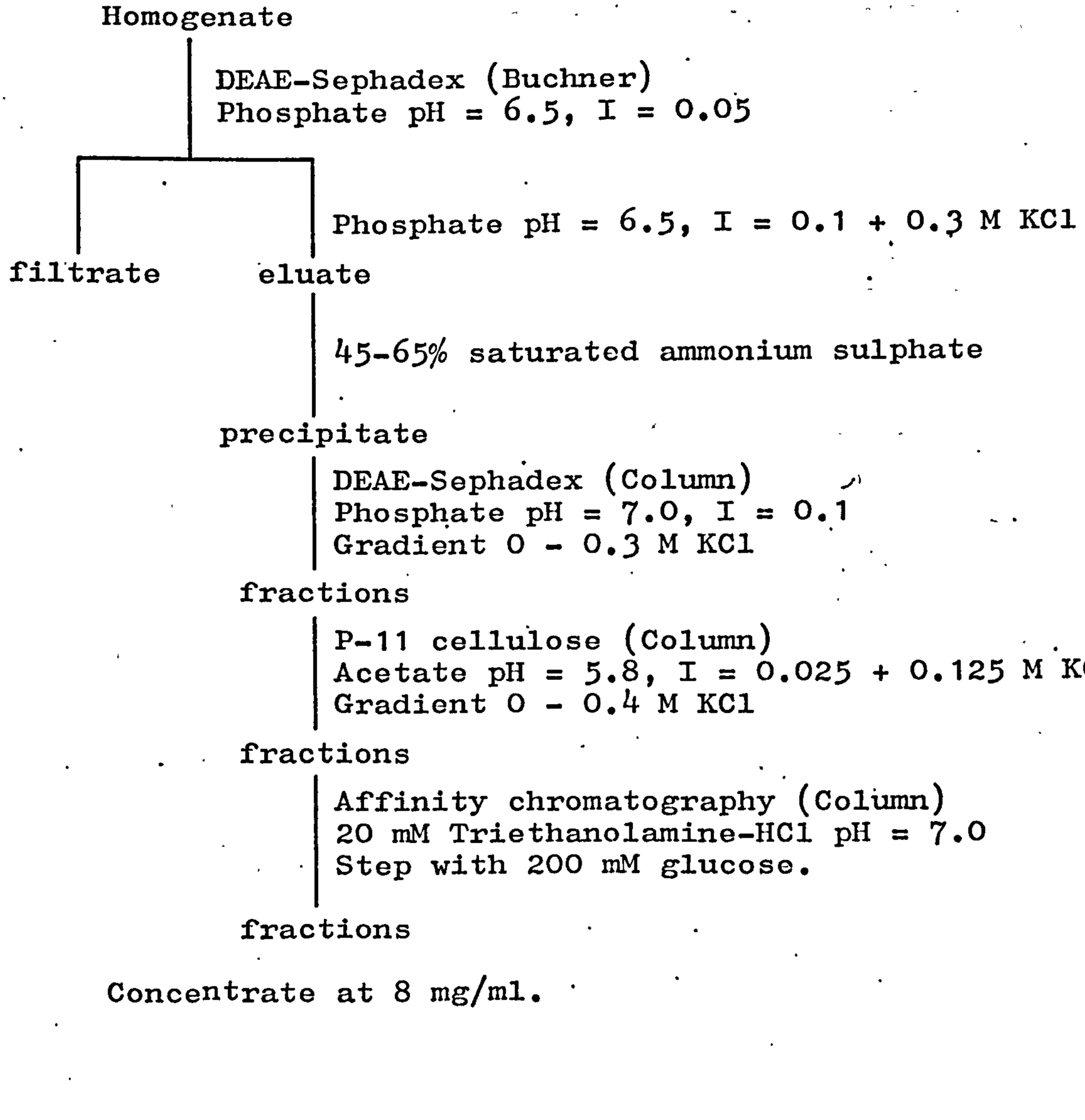


Table 5.1. Summary of a typical purification procedure for hexokinase from heart

	Step	Volume	Protein	Activity	Specific Activity	Purification	Yield
		ml	mg	Units	Units/mg	-fold	%
1.	Homogenate	5,900	88×10^3	3,050	0.035	1	100
2.	DEAE-Sephadex (Buchner)	6,200	15×10^3	3,000	0.20	5.7	98
3.	Ammonium sulphate 45 - 65% satd.	100	5.1×10^3	2,400	0.47	13.5	79
4.	DEAE-Sephadex (Column)	115	1.4×10^3	2,400	1.70	49	79
5.	P-11 cellulose (Column)	188	63	1,750	27.7	790	57
6.	Affinity chromatography*	79	25	1,450	58.0	1660	47

* For this step the protein concentration was estimated using an absorption coefficient, $A_{280}^{1\%}$, of 6 determined by the method of Babul and Stellvagen (1966).

Buchner funnel was about 5 litres per hour. The resin in each funnel was washed with the above buffer until the filtrate was colourless, applying mild suction during the final washes. The enzyme was eluted with phosphate buffer pH = 6.5, I = 0.1 plus 0.3 M KCl using 2.5 lt of buffer for each Buchner funnel, the resin being stirred and sucked dry during the final stages of elution. The total weight of dry resin used was 150 g.

Step 3. Fractionation with ammonium sulfate.

To each litre of eluate 277 g of ammonium sulfate were added to give a 45% saturation with the salt. The precipitate was allowed to form on overnight storage. The suspension of the 45% saturated ammonium sulfate was centrifuted at 10,000 x g for 20 minutes using a Sorvall RC2-B centrifuge. The precipitate was discarded and the supernatant was made 65% saturated in ammonium sulfate by the addition of 134 g of salt per 1 of solution. The precipitate was allowed to form on storage and the suspension was centrifuged as above. The supernatant was discarded and the precipitate was dissolved in phosphate buffer pH = 7.0, I = 0.1 and dialysed against 60 volumes of the same buffer. The dialysis buffer was replaced once.

Step 4. Chromatography on DEAE - Sephadex A-50.

Any precipitate remaining after dialysis was removed

by centrifugation at 15,000 x g for 10 minutes. The solution was applied to a 30 x 2.7 cm column equilibrated with phosphate buffer pH = 7.0, I = 0.1. A linear salt gradient was applied using two identical interconnected 250 ml vessels, one containing the phosphate buffer pH = 7.0, I = 0.1, the other containing the same buffer plus 0.3 M KCl. The flow rate was 30 ml/hour and 5 ml fractions were collected. The elution profile is given in Figure 5.2. Fractions were pooled as shown and were dialysed against 60 volumes of acetate buffer pH = 5.8, I = 0.15 with two changes.

Step 5. Chromatography on P-11 cellulose.

The dialysed solution was centrifuged at 15,000 x g for 10 minutes to remove any precipitate formed during dialysis and was applied to a 30 x 2.7 cm column equilibrated with acetate buffer pH = 5.8, I = 0.025 + 0.125 M KCl. A linear gradient was applied using two identical interconnected 250 ml vessels, one containing the above acetate buffer and the second containing the same buffer plus 0.4 M KCl. The flow rate was 50 ml/hour and 5 ml fractions were collected. The elution profile is given in Figure 5.3. Fractions were pooled as shown and were concentrated with vacuum dialysis to 10 ml and dialysed against 300 volumes of 20 mM Triethanolamine-HCl buffer pH = 7.0,

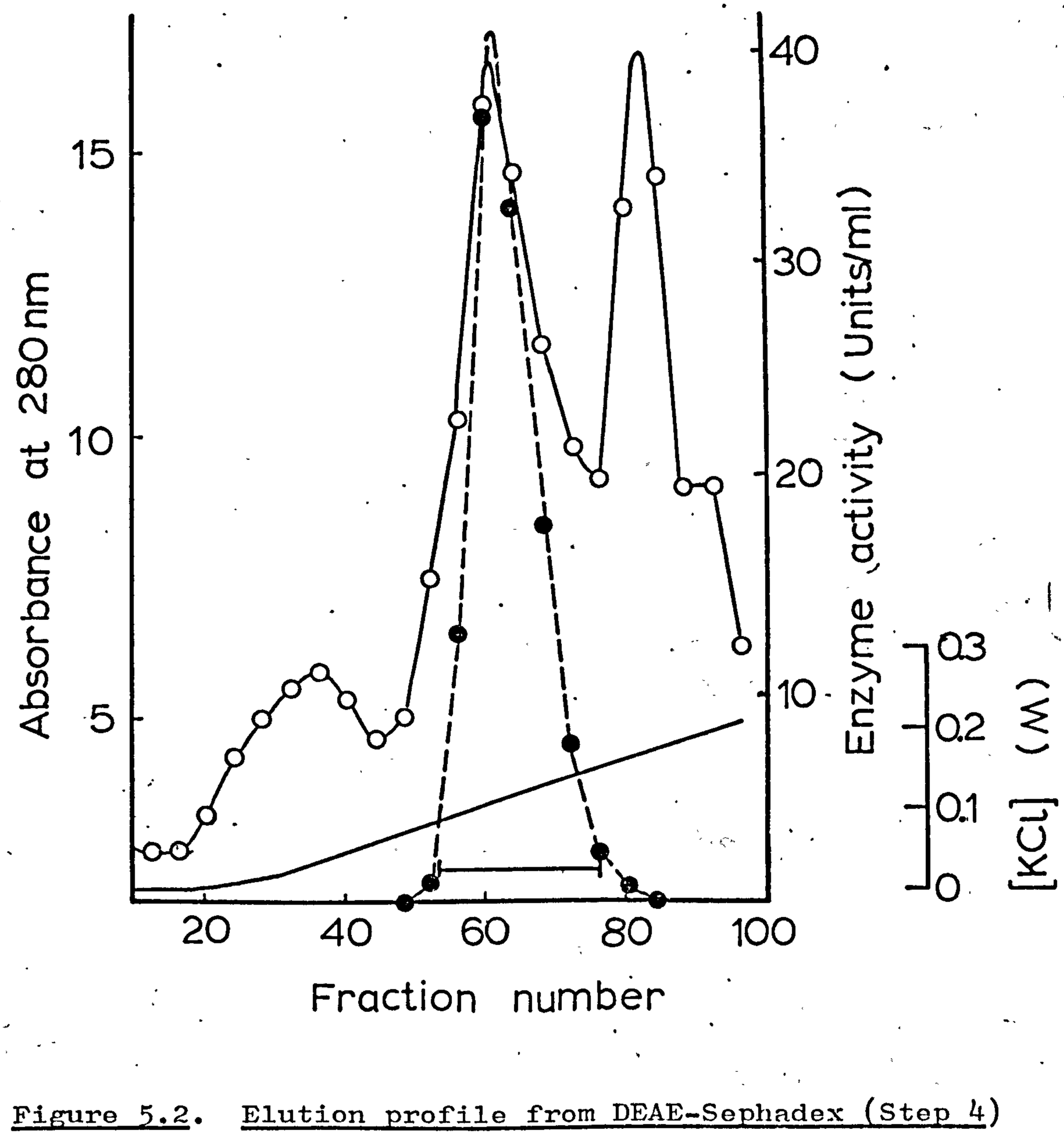


Figure 5.2. Elution profile from DEAE-Sephadex (Step 4)

(○—○) Absorbance at 280 nm; (●—●) Enzyme activity. Fractions of 5 ml were collected. The horizontal line indicates the fractions pooled after elution.

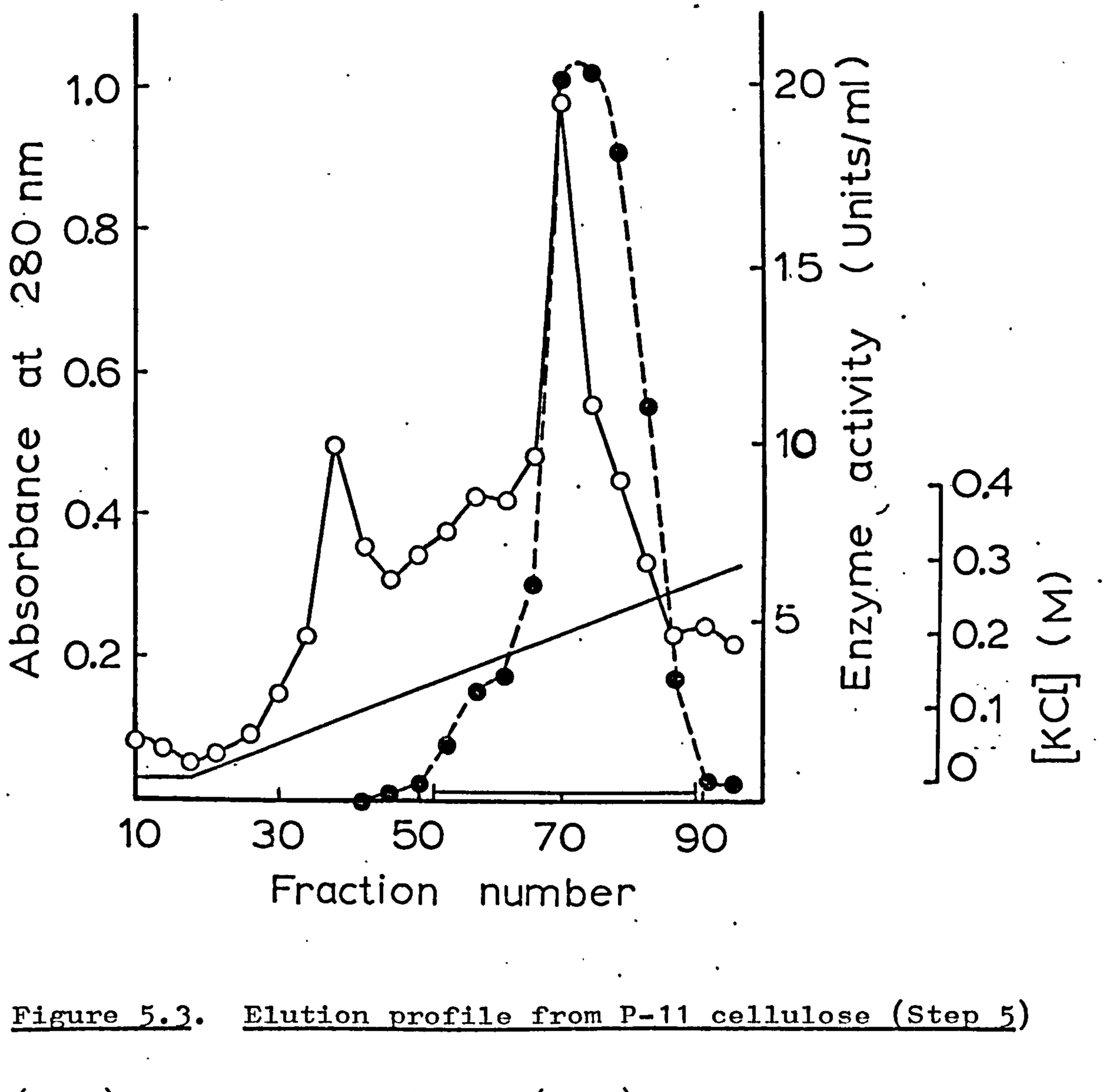


Figure 5.3. Elution profile from P-11 cellulose (Step 5)

(○—○) Absorbance at 280 nm; (●—●) Enzyme activity. Fractions of 5 ml were collected. The horizontal line indicates the fractions pooled after elution.

without glucose. The dialysis buffer was replaced once.

Step 6. Affinity chromatography.

The dialysed solution was centrifuged at 15,000 x g for 10 minutes to remove any precipitate and the solution was applied to a 19 x 2.6 cm affinity column containing immobilised glucosamine on Sepharose 4B. The resin was equilibrated with 20 mM Triethanolamine-HCl buffer pH = 7.0. A volume of 125 ml of the same buffer was used to wash out proteins not bound on the resin. The subsequent passage of 160 ml of the same buffer containing 50 mM KCl released a protein peak. Finally the hexokinase activity peak was eluted with the previous buffer containing 200 mM glucose. The elution profile is given in Figure 5.4. The flow rate was adjusted to 35 ml/hour with a peristaltic pump and 4.6 ml fractions were collected. Fractions of hexokinase activity were pooled as shown and were concentrated with vacuum dialysis to a small volume with a protein concentration of about 8 mg/ml and dialysed against 300 volumes of phosphate buffer pH = 7.0, I = 0.1. The enzyme was stored at 0-4°C under nitrogen.

(c) Comments on affinity chromatography

The affinity of an enzyme to a ligand is altered upon the immobilization of the latter. Reasons for this change

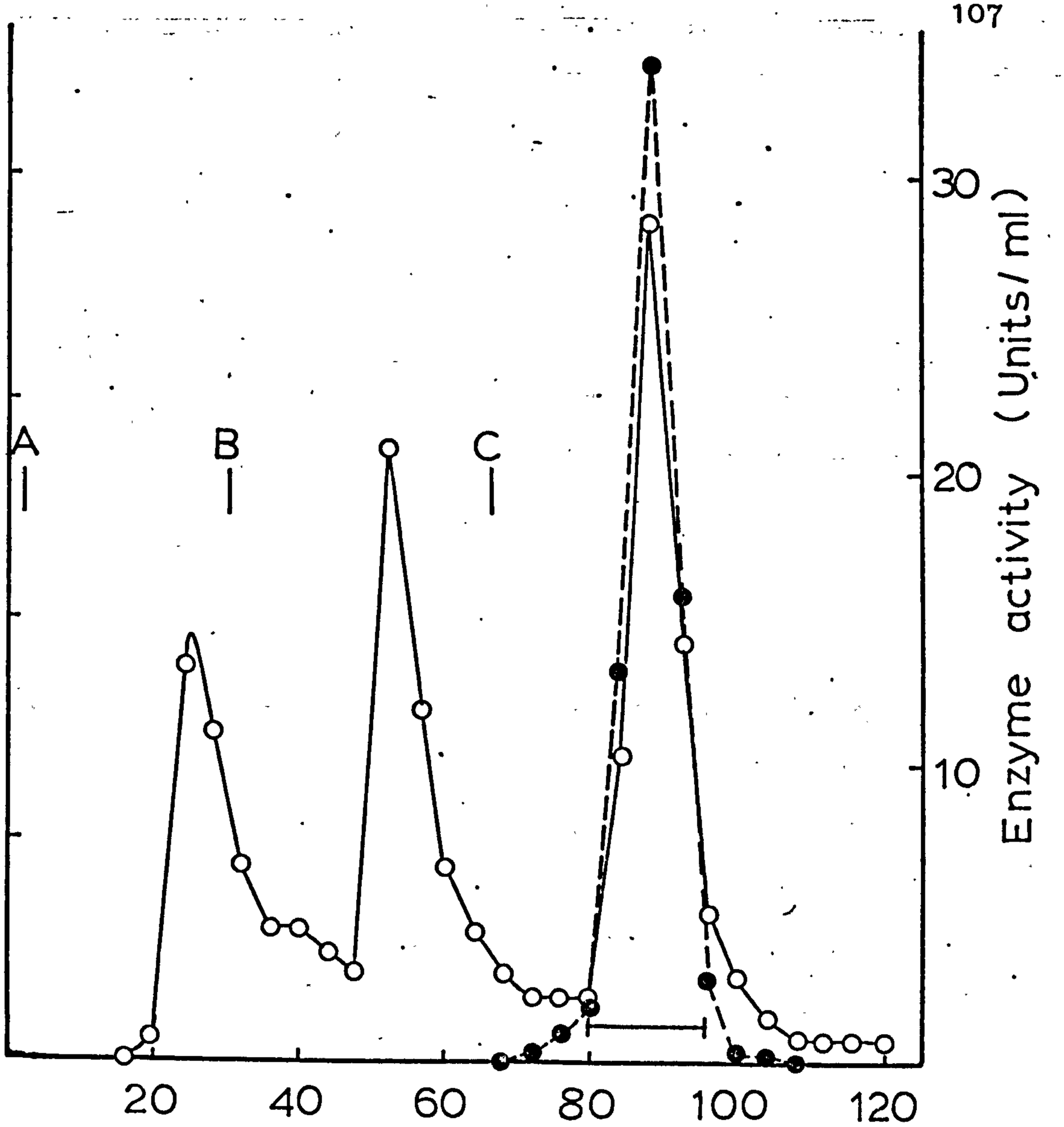


Figure 5.4. Elution profile from the affinity chromatography (Step 6)

(O—O) Absorbance at 280 nm; (●—●) Enzyme activity.

Fractions of 4.6 ml were collected. The horizontal line indicates the fractions pooled after elution.

A : 20 mM Triethanolamine-HCl buffer pH = 7.0

B : Buffer A with 50 mM KCl added

C : Buffer B with 200 mM glucose added.

are the chemical modification of the ligand for its binding on the insoluble matrix and its' steric availability to the enzyme. Other factors affecting the affinity are directly related to the ligand and enzyme such as: the temperature (Harvey et al., 1974b), the pH (Lowe et al., 1974a), the ionic strength and hydrophobicity of the liquid phase (Lowe and Mosbach, 1975), the geometry of the column, the input flow rate of the enzyme solution (Lowe et al., 1974b), and the concentration of the immobilized ligand in the resin (Harvey et al., 1974a; Holroyde et al., 1976a).

The response of hexokinase from various sources to immobilized mucleotide and glucose derivatives is summarized in Table 5.2. Glucosamine, is phosphorylated by brain hexokinase while N-acetylglucosamine is not phosphorylated and acts as a competitive inhibitor of this enzyme with respect to glucose (Harpur and Quastel, 1949; Bachelard et al., 1971). The derivative N-(6-Aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose was found to be a competitive i hibitor of rat liver glucokinase versus glucose (K_i = 0.75 mM) by Holroyde et al., (1976a). Glucosamine bound directly to Sepharose had no affinity for liver glucokinase (Holroyde et al., 1976a). However the interposition of a six carbon spacer arm promoted binding of both rat liver glucokinase (Holroyde et al., 1976b) and rat skeletal muscle HK II (Holroyde and Trayer, 1976), and so allowed the isolation of these two enzymes.

Table 5.2. Interaction of mammalian hexokinases with immobilized ligands

Ligand	Enzyme	Interaction	Reference
N^6-(6-Aminohexyl)-ADP	Rat liver hexokinase	+	Trayer et al., 1974
"	Rat liver glucokinase	+	"
8-(6-Aminohexyl)amino-ADP	Rat liver hexokinase	-	"
"	Rat liver glucokinase	+	"
N-(6-Aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose	Rat liver glucokinase	+	Holroyde et al., 1976b
"	Rat muscle HK II	+	Holroyde and Trayer, 1976
D-glucosamine bound to CH-Sepharose 4B	Human erythrocyte	+	Rijksen and Staal, 1976a

A resin prepared by coupling glucosamine to activated CH-Sepharose 4B (Sepharose 4B with a six-carbon spacer arm) helped in the purification of human erythrocyte hexokinase by retardation of hexokinase activity towards the bulk of the proteins of the enzyme sample applied (Rijksen and Staal, 1976a).

Trayer et al. (1974) comment that hexokinase (with low Km for glucose) from rat liver binds to immobilized N^6-(6-aminohexyl)-ADP, and not to immobilized 8-(6-amino-hexyl)-amino-ADP. In contrast, glucokinase binds to both nucleotides (Trayer et al., 1974). This suggests that the binding of hexokinase to these nucleotides is weak and depends on the mode of attachment to the matrix.

Interaction of human heart and erythrocyte hexokinase with immobilized glucosamine

The preparation of an affinity resin using Sepharose 4B, 6-aminohexanoic acid (as spacer) and glucosamine (as the ligand) is described in Chapter 2. Pilot experiments were performed by using small columns (10 x 0.8 cm) equilibrated with 20 mM Triethanolamine-HCl buffer pH = 7.0 containing 7.5 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol and 50 mM KCl at 4^oC. The flow rate was kept constant at 10 ml/hour. About 1.5 units of purified heart hexokinase in 1 ml of the above buffer was

applied on a column. No activity passed through in 5 bed volumes of washing solution. Application of a 60 ml linear gradient 0-0.3 M KCl eluted a single peak corresponding to a KCl concentration of 150 mM. Leaking of enzyme activity began at 100 mM KCl. Alternatively hexokinase was eluted with a pulse of 200 mM glucose. The latter method of elution was adopted for its better recovery and for its specificity.

Non specific binding was tested by using resin coupled to 6-aminohexanoic acid alone (without further attachment of glucosamine). The conditions of the experiment performed on this resin were as those used for the normal affinity step for the purification of heart hexokinase, and the hexokinase preparation used was that recovered from the preceding step. This control-resin did not retain any hexokinase activity and no improvement of the specific activity was obtained.

The affinity resin interacts specifically with both the heart and the erythrocyte enzyme, as can be seen from the facts that both enzymes bind on the resin, are eluted with glucose and that at least one of them (heart) does not interact with the matrix and the spacer. However this resin lacks chemical characterization, as compared with resin prepared by Holroyde et al. (1976a), that is by coupling of pre-synthesized N-(6-Aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose to activated Sepharose 4B. However the relatively complicated and time consuming presynthesis

of the glucosamine derivative was avoided with the present method.

The effectiveness of the resin diminished considerably after one year of storage at 4°C with sodium azide, as seen from the fact that hexokinase was eluted as a retarded peak as compared to the main protein peak, without the pulse of glucose. This deteriorated resin behaved as the affinity resin reported by Rijksen and Staal (1976a) for the purification of erythrocyte hexokinase. The most possible explanation is that the decrease of the affinity of the enzyme for the resin was caused by a decrease of the concentration of the ligand bound to the resin.

Sepharose 4B with immobilized N^6-[(6-Aminohexanoyl)carbamoylmethyl]ATP

The preparation of this resin is described in Chapter 2. Small columns of 10 x 0.8 cm were equilibrated with 50 mM Tri-HCl buffer pH = 8.1 containing 10 mM $MgCl_2$, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Chromatography was performed at room temperature with a constant flow rate of 15 ml per hour. Samples of 2-5 units of purified heart and commercial yeast hexokinase in 2-10 ml of the above buffer were applied to the column. Both hexokinases passed through with 15 and 40% recoveries for the heart and the yeast enzyme respectively. Pulses with 10 mM

$MgCl_2$ plus 5 mM ATP or 1 M KCl did not elute any more activity. Yeast hexokinase was tested since it was reported by Lindberg and Mosbach (1975) that it dephosphorylated this immobilized ATP analogue in the presence of Mg^{2+} and glucose. The very low recovery of heart hexokinase may be explained by its' instability in the absence of glucose at room temperature.

Several explanations for the non-binding of heart hexokinase may be given:

(i) The enzyme has no affinity for this ATP analogue because of the modification of the ATP molecule for immobilization, or its' lower availability to the enzyme on attachment to the resin;

(ii) The enzyme has small affinity for this ATP analogue so that a low concentration of the analogue in the resin may exclude binding. This is likely as the estimated maximum concentration of the ATP-analogue on the resin is in the range of the K_m for the true substrate (MgATP);

(iii) The reaction mechanism of heart hexokinase is an ordered one, with glucose binding first. Against this view is the fact that some investigators have proposed a rapid equilibrium random mechanism for mammalian hexokinases (Bachelard et al., 1971; Gerber et al., 1974), while Toews (1966) has proposed an ordered sequential mechanism

but with ATP binding first.

(d) Other comments on the purification procedure

A small scale purification was tried for heart hexokinase with exactly the same procedure as described in Chapter 4 for placental hexokinase. The specific activity after Step 2 (dialysis) was more than two-fold higher and so was the specific activity of the purified 2 mg of protein after Step 5 (gel filtration) i.e. 20 units/mg as compared with 8.6 units/mg from placenta. In this procedure the extraction was in acetate buffer pH = 5.6, I = 0.1 + 0.2 M $MgCl_2$. The subsequent chromatographic step requires dilution of this buffer. This involves excessively large volumes when using large amounts of tissue. Extraction with phosphate buffer pH = 7.5, I = 0.02 (in the method adopted) gave the same specific activity for hexokinase (2.65 units/g tissue) as the acetate buffer with $MgCl_2$ (2.2 units/g tissue). The extract with phosphate buffer required no further dilution for chromatography.

If in the procedure summarised in Figure 5.1., gel filtration (Sephadex G-200) was used instead of the affinity chromatography step, a specific activity of 47 units/mg was obtained ($A^{1\%}_{280\ nm}$ = 10). If gel filtration was used after the affinity chromatography step, one peak of

enzyme activity was observed coinciding with the sole protein peak (Chapter 9) and no increase in the specific activity was obtained. Thus the gel-filtration step was judged to be unnecessary.

One peak of enzyme activity was found from DEAE-Sephadex A-50 and Whatman P-11 cellulose chromatographic steps. However a trace of activity at the leading edge was consistently found on P-11 cellulose. Chromatographic fractions assayed with glucose concentrations of 0.5 mM and 100 mM respectively did not reveal the coexistence of the HK III or HK IV with the reported HK I for human heart.

(e) Solubilisation of particulate hexokinase

In order to test the effect of pH and salts on the solubilisation of particulate hexokinase, the following experiments were performed. Human heart tissue stored at $-45^{o}C$ was thawed, chopped and minced. A quantity of the minced tissue was homogenised with 3 volumes of chilled phosphate buffer pH = 7.5, I = 0.02 in a MSE Atomix blender for three, 20 second intervals at full speed. The homogenate was centrifuged at 40,000 x g for 30 minutes in a Sorvall RC2-B centrifuge. The supernatant was decanted and the pellet was kept overnight on ice. Quantities of 2 g were dispersed in 6 ml of the various buffers used for

the solubilization experiments. The dispersion was facilitated by agitation using a Whirlmixer (Fisons) for 1 minute. After 2 hours of incubation on ice the homogenates were centrifuged as above and the supernatants were assayed for enzyme activity.

The effect of buffered solutions of I = 0.1 at pH values from 5.4 to 7.9 is shown in Figure 5.5. The solubilisation of particulate hexokinase was unexpectedly increased by increasing pH, reaching a plateau at approximately pH = 7.0. Previous investigations for porcine heart hexokinase by Hernandez and Crane (1966) and by Easterby and O'Brien (1973) showed an inverse relation i.e. decrease of solubilisation by increasing the pH in the range of 5 to 9 and 5.6 to 7.1 respectively. The use of fresh tissue in these reports may account for the difference from the present results on stored material.

In accordance with both reports the solubilisation increased with increasing concentration of KCl and the effect was more pronounced at lower pH values (Easterby and O'Brien, 1973). Results are shown in Figure 5.6. The effect of salt concentration seems to be that of ionic strength rather than of the actual concentration of ions since KCl (monovalent cation) and $MgCl_2$ (divalent cation) on an ionic strength basis had similar solubilising effects (Figures 5.6, (a) and (b) respectively).

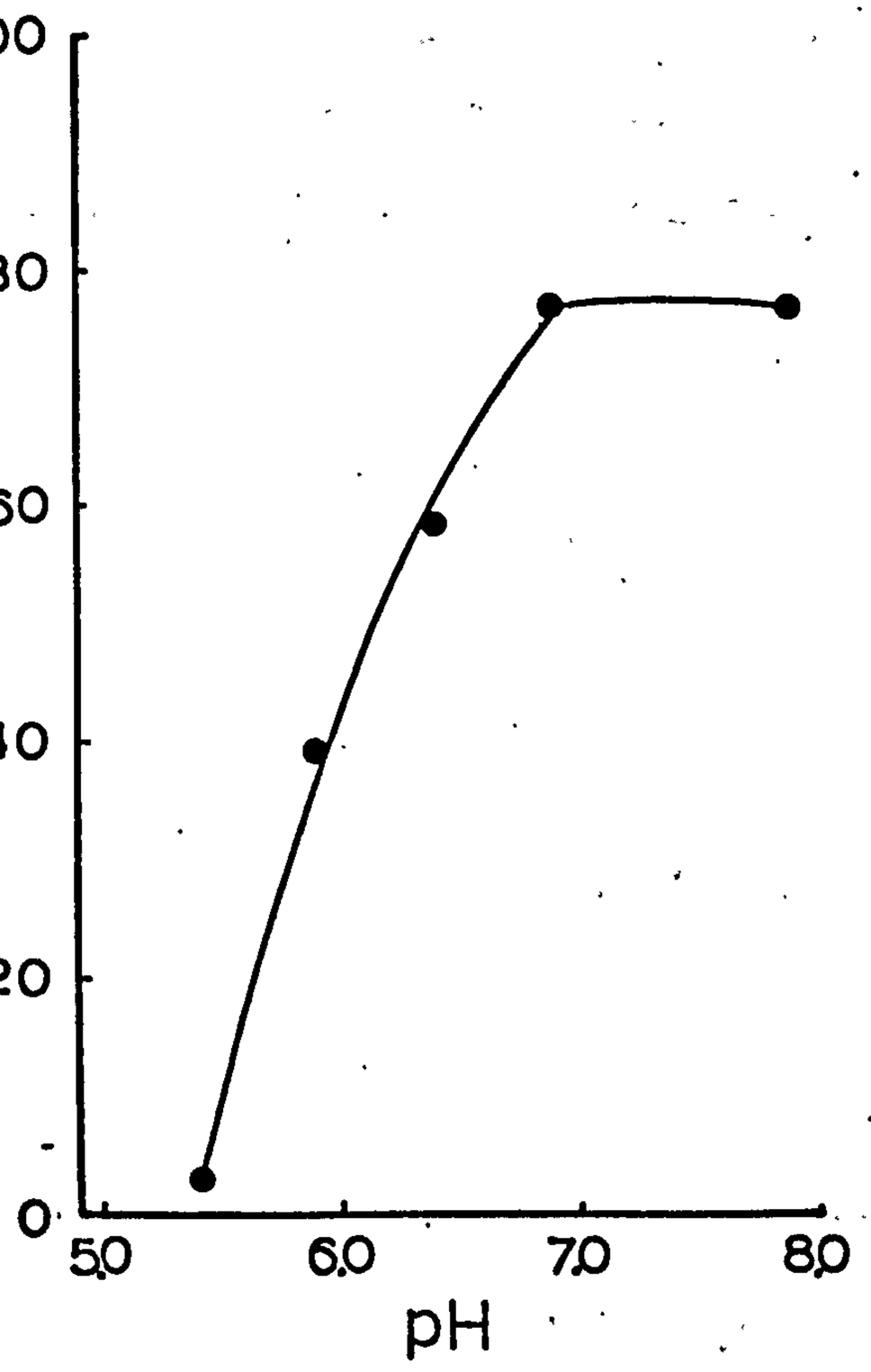


Figure 5.5. Effect of pH on the solubilisation of hexokinase

Solubilisation is shown as a percentage of the maximum obtainable with phosphate buffer pH = 5.9, I = 0.1 plus 0.4 M KCl.

Acetate buffer was used for pH = 5.4, the rest were phosphate buffers. The ionic strength of all buffers was 0.1.

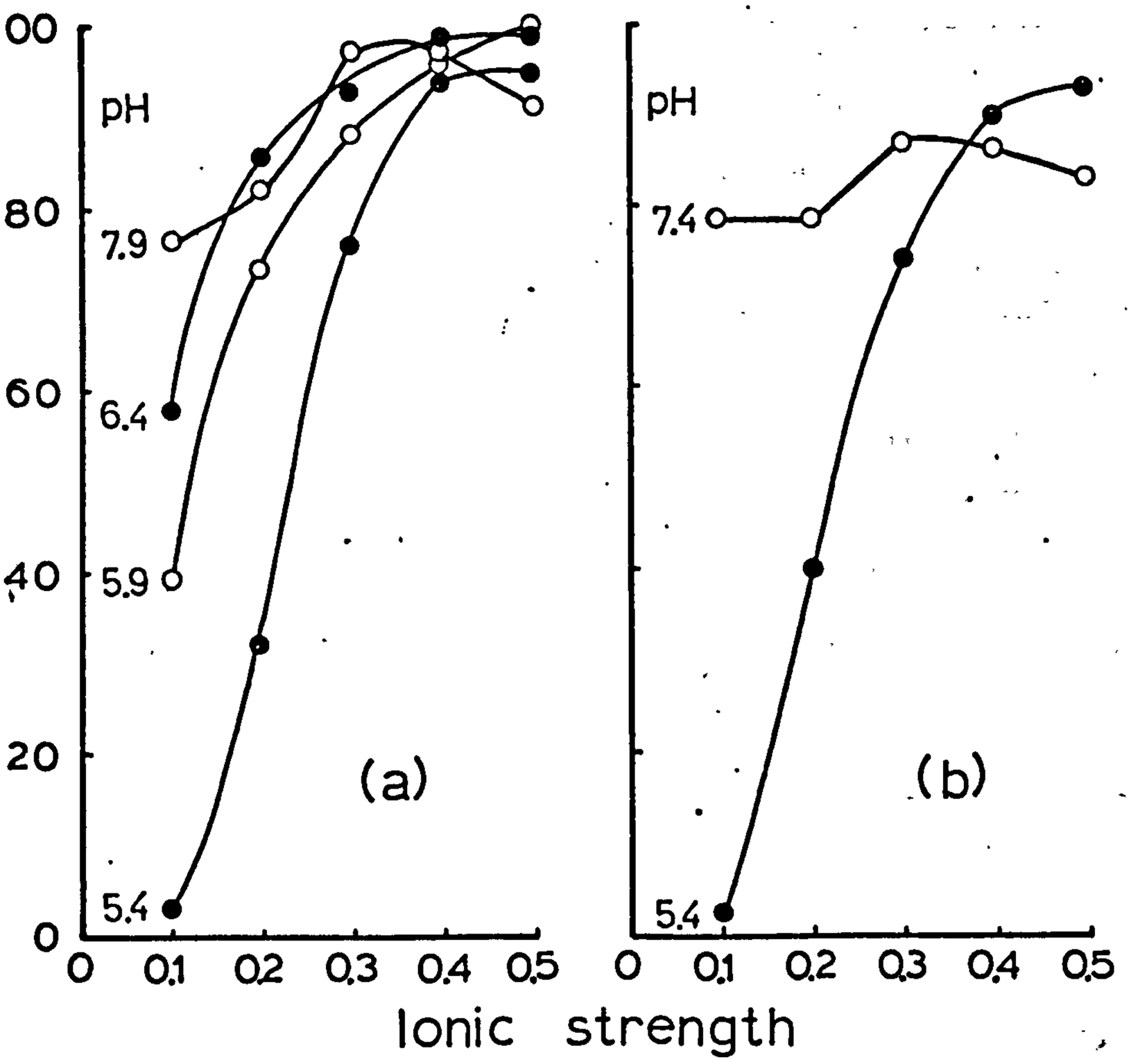


Figure 5.6. Effect of ionic strength at various pH values on the solubilisation of hexokinase

Solubilisation is shown as a percentage of the maximum obtainable with phosphate buffer pH = 5.9, I = 0.1 plus 0.4 M KCl.

All buffers used had I = 0.1. Higher values of I were obtained by the addition of KCl (a) and $MgCl_2$ (b).

The following buffers were used: acetate, pH = 5.4; phosphate, pH = 5.9; phosphate, pH = 6.4; phosphate, pH = 7.9; Tris-HCl, pH = 7.4.

(f) Conclusions

The purification procedure developed for human heart hexokinase contains 6 steps and takes 12 days. Beginning with 1.15 Kg of minced tissue, 25 mg of protein was purified with a specific activity of 58 units/mg. The overall purification was 1,700 fold and the overall yield was 47%.

Reported purifications of mammalian low K_m hexokinases are summarized in Table 5.3. However only the purifications of porcine heart hexokinase by Easterby and O'Brien (1973) and that of rat brain hexokinase by Chou and Wilson (1972) are supported by solid evidence of homogeneity.

One previous purification of human heart hexokinase has been reported by Neumann et al. (1974). That purification procedure had certain disadvantages. The overall yield was very low (2.5%) so that an initial quantity of 8 Kg of heart tissue had to be processed over 7 steps (the first three of them repeated four times) for the preparation of 10 mg of purified protein. The material was tested for purity only with immunoelectrophoresis and showed only one precipitation arc. The comparatively high specific activity (71 units/mg) of the purified material should be ascribed mainly to the higher pH of the assay solution used and to a lesser extent to the lower ionic strength. Both of these factors affect the maximum veloc-

Table 5.3. Summary of purifications of low K_m mammalian hexokinases

Reference	Source	Specific Activity	No of Steps	Yield	Purified Protein	Method of protein measurement	Assay Solution	Temperature
		Units/mg		%	mg			°C
Neumann et al., (1974)	Human heart	71	7	2.5	10.2	Biuret	74 mM Tris-HCl, pH=7.4 10 mM ATP, 20 mM $MgCl_2$, 25 mM glucose 0.5 Units/ml G6PD, 0.55 mM NADP	25
Easterby and O'Brien (1973)	Porcine heart	82	5	20	8	$A^{1\%}_{280\ nm}$= 6.0	50 mM Tris-HCl, pH=7.6 10 mM ATP, 20 mM $MgCl_2$, 3 mM glucose 0.2 Units/ml G6PD, 0.1 mM NADP	30
Holroyde and Trayer (1976)	Rat skeletal muscle	210	5	48	2.2	Turbidimetric microtannine	50 mM Tetramethylammonium-glycylglycinate pH = 8.0, 5 mM ATP, 5 mM $MgCl_2$ 0.5 mM glucose excess of G6PD, 1 mM NADP	30

cont./

Reference	Source	Specific Activity (U/mg)	No of Steps	Yield (%)	Purified Protein (mg)	Method of Protein measurement	Assay Solution	Temperature
Chou and Wilson (1972)	Rat brain	60	4	51	.4	Turbidimetric micromethods	40 mM HEPES, pH=7.5, 6.7 mM ATP, 6.7 mM $MgCl_2$, 3.3 mM glucose, 1 Unit/ml G6PD, 0.32mM NADP	25
Redkar and Kenkare (1972)	Bovine brain	83	7	18	22	Lowry	80 mM Tris-HCl, pH = 7.4, 6 mM ATP, 8 mM $MgCl_2$ 27 mM glucose, 0.4 Units/ml G6PD, 0.16 mM NADP	30
Schwartz and Basford (1967)	Bovine brain	80	4	28	18.7	Lowry	80 mM Tris-HCl, pH = 8.0, 15 mM ATP, 19 mM $MgCl_2$ 25 mM glucose, 3 µg/ml G6PD, 1 mM NADP	30
Present results	Human heart	58	6	47	25	$A^{1\%}_{280\ nm}$ = 6.0	130 mM TES-KOH, pH = 7.2, 5 mM ATP, 10 mM $MgCl_2$ 5 mM glucose, 0.25 Units/mg G6PD, 0.6 mM NADP, 75 mM KCl.	25

ity of the hexokinase reaction as shown in Chapter 6. A sample assayed according to this assay method (Neumann et al., 1974) had a 70% greater activity than the assay method used in the present work (Chapter 2). A corresponding assay according to the method of Easterby and O'Brien (1973) gave an activity value 120% higher than in the present work.

The homogeneity of the purified hexokinase described in this Chapter is discussed in Chapter 9.

SECTION 3

Enzyme activity

CHAPTER 6

Kinetics of human heart hexokinase.

(a) Measurement of enzyme activity

The kinetics of the coupled assay for hexokinase in the presence of G6PD have been studied extensively (Easterby, 1973; Storer and Cornish-Bowden, 1974). By using excess of G6PD (0.5 units per ml of assay solution) the transition time was kept small (calculated to be less than 10 seconds) and the steady state of glucose 6-phosphate 0.2 μM, calculated according to Easterby (1973), was much lower than the reported Ki of glucose 6-phosphate versus glucose (77 μM) for human placental hexokinase (Gustke, 1975).

Reaction velocities were calculated from the change in absorbance at 340 nm measured by a Perkin-Elmer 124 spectrophotometer connected to a 64 chart recorder. All measurements were performed at 25°C by circulating water to the cuvette holder from a waterbath. Cuvettes of 1 cm light-path and 3 ml capacity were used. Initiation of the hexokinase reaction was made by the addition of 20 μl of a diluted (glucose free) sample of purified hexokinase.

(b) The effect of pH on activity

The ionic strength of the buffers used was kept constant since, as shown later, hexokinase activity decreases with increasing ionic strength. The reaction mixture contained 100 mM glucose, 5 mM ATP, 10 mM $MgCl_2$, 0.6 mM NADP and 0.5 units/ml G6PD in the appropriate buffers of 0.05 ionic strength.

The profile of hexokinase activity over a pH-range from 4.5 to 8.8 is shown in Figure 6.1. The activity increased gradually from pH = 4.5 to pH = 7.7. Above this pH the activity remained constant up to the tested pH = 8.8.

This profile is in accordance with pH values for maximal activity reported for mammalian hexokinases (Table 6.1).

(c) The effect of salts on activity

The assay solution used contained 100 mM glucose, 5 mM ATP, 10 mM $MgCl_2$, 0.6 mM NADP and 0.5 units/ml G6PD in Tris-HCl buffer pH = 8.0, I = 0.01.

The effect of various concentrations of the salts, KCl, NaCl, NH_4Cl, K_2HPO_4 and the base Tris is shown in Figures 6.2 and 6.3. The pH of the assay solution varied little because of the addition of salts, i.e. between 8.0 and 8.3 (well inside the pH optimum).

All salts studied showed a similar effect of decreasing the maximal hexokinase activity with increasing ionic

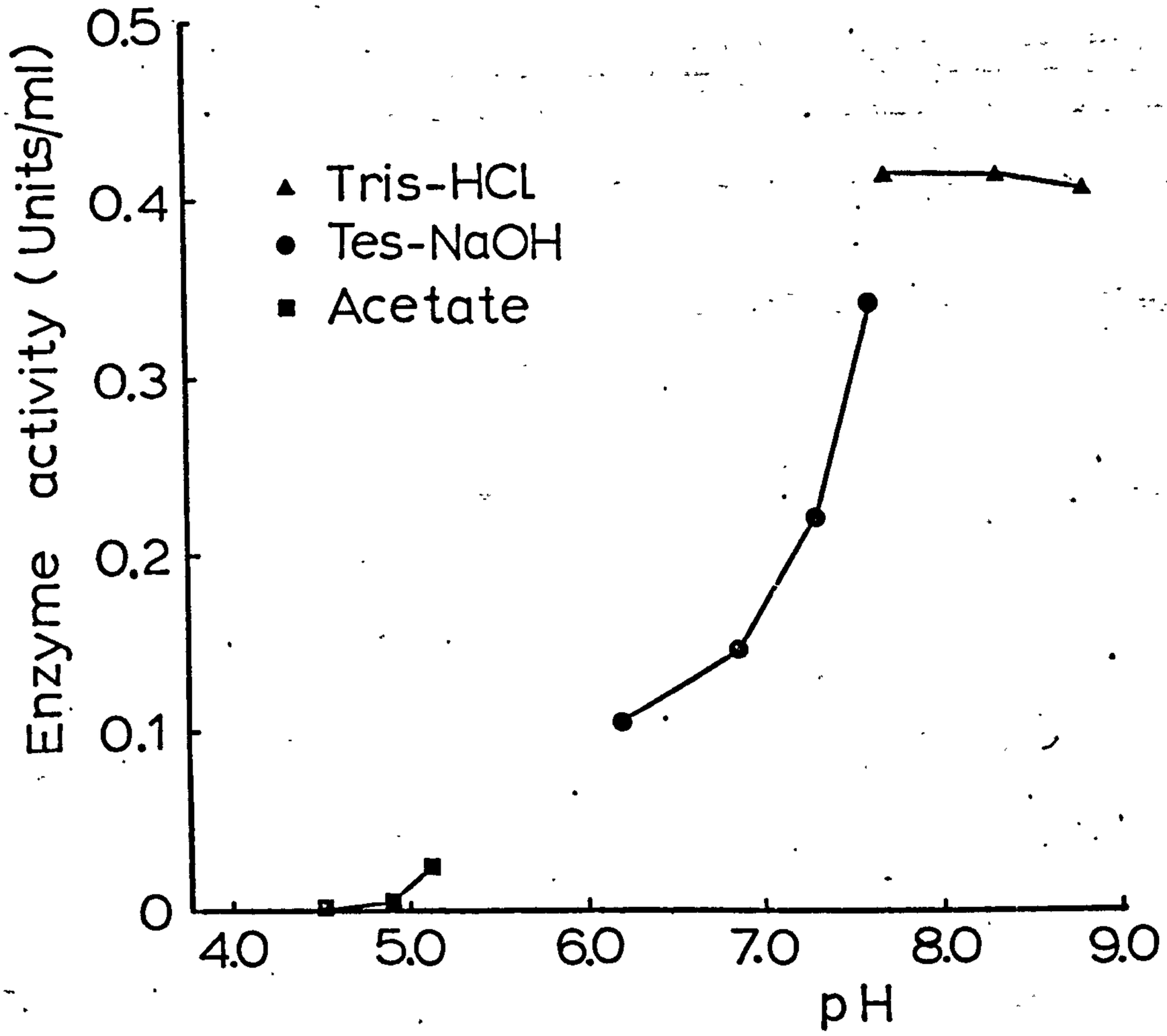


Figure 6.1. The dependence of heart hexokinase activity on pH, using buffers of I = 0.05.

Table 6.1. pH optima of Vmax for low Km mammalian hexokinases

Tissue	pH	Reference
Human erythrocytes	7.0-8.4	Rijksen and Staal (1976a)
Human erythrocytes	8.1	Chapman et al., (1962)
Calf heart	8.0-9.0	Sols and Crane (1954)
Dog heart	8.0-9.0	Mayer et al., (1966)
Rat tissues (HK I to HK III)	7.8-8.8	Grossbard and Schimke (1966) Schimke and Grossbard (1968)
Rat liver (HK I to HK III)	8.0-9.0	González et al., (1967).

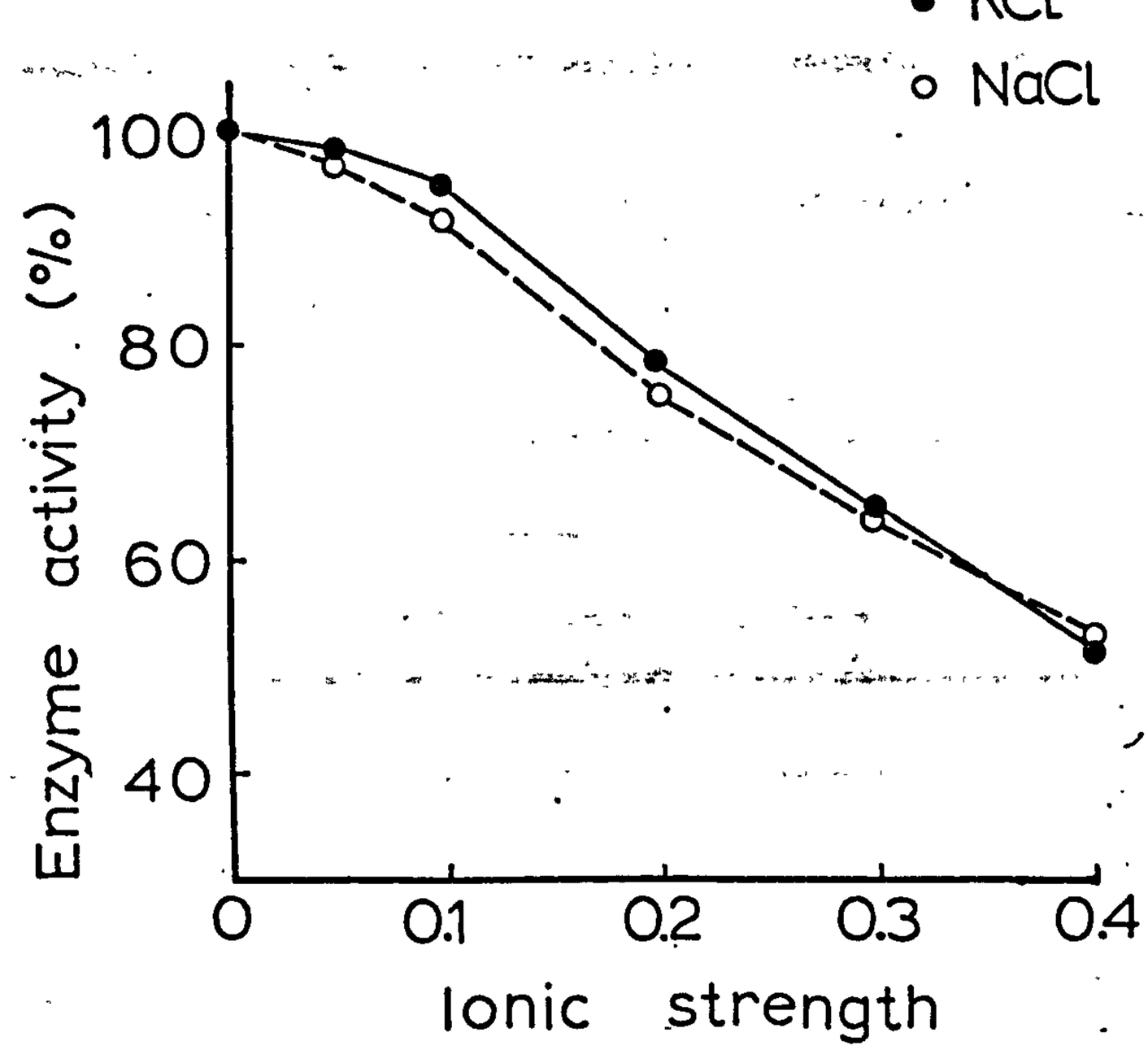


Figure 6.2. The effect of KCl and NaCl on the activity of heart hexokinase at pH = 8.0 in a low ionic strength Tris-HCl buffer

An activity of 100% was taken as that in Tris-HCl buffer, ionic strength 0.01.

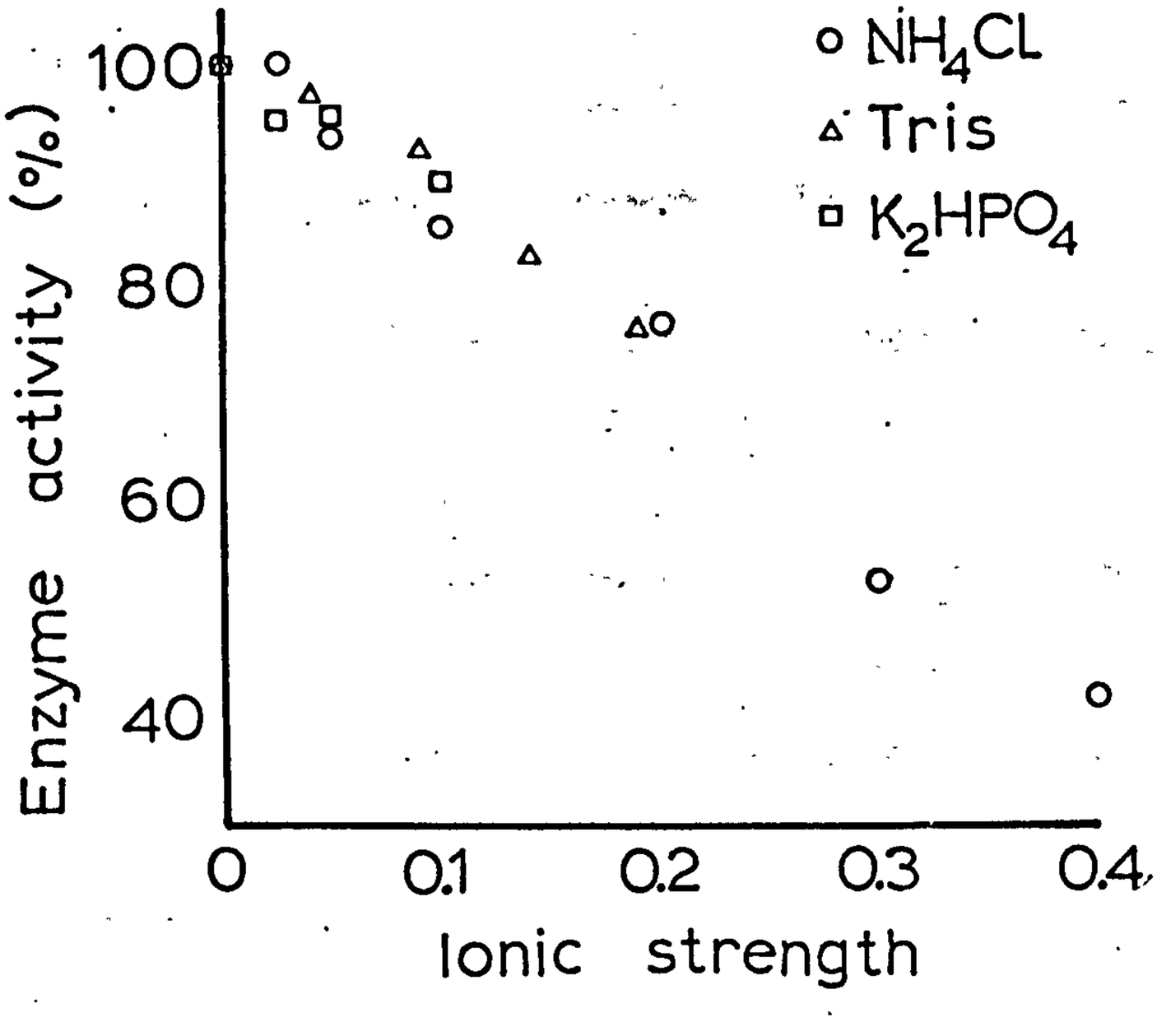


Figure 6.3. The effect of salts on the activity of heart hexokinase at pH = 8.0 in a low ionic strength Tris-HCl buffer

An activity of 100% was taken as that in Tris-HCl buffer, ionic strength 0.01.

strength. A similar effect is reported for rat skeletal muscle hexokinase (Weiser et al., 1970).

(d) Kinetic constants for glucose and $MgATP^{2-}$

For the measurements of Michaelis constants for glucose and $MgATP^{2-}$, assay solutions were prepared containing 0.6 mM NADP and 0.5 units/ml G6PD in TES-KOH buffer pH = 7.0, I = 0.065.

Glucose concentration was varied from 20 to 100 μM, $MgATP^{2-}$ concentration was varied from 0.2 to 1.0 mM by the addition of calculated amounts of $MgCl_2$ and ATP. The concentration of Mg^{2+} ions was held constant at 2 mM.

Under the above conditions the rest of the ion species originating from Mg^{2+} and ATP^{4-} varies as follows:

$[ATP^{4-}]$ from 5 to 25 μM,
$[ATPH^{3-}]$ from 3 to 15 μM and
$[ATPHMg^{-}]$ from 3 to 16 μM.

Calculations were based on the following association and dissociation constants:

(i) $Mg^{2+} + ATP^{4-} \overset{K_1}{\rightleftharpoons} MgATP^{2-}$, $K_1 = 4 \times 10^4\ M^{-1}$

(ii) $Mg^{2+} + ATPH^{3-} \overset{K_2}{\rightleftharpoons} MgHATP^{-}$, $K_2 = 5.8 \times 10^2\ M^{-1}$

(iii) $ATPH^{3-} \overset{K_3}{\rightleftharpoons} ATP^{4-} + H^{+}$, $K_3 = 8.1 \times 10^{-8}\ M$

(iv) $ATPHMg^{-} \overset{K_4}{\rightleftharpoons} ATPMg^{2-} + H^{+}$, $K_4 = 6 \times 10^{-6}\ M$.

reported by Phillips et al. (1966).

The reasons for keeping the Mg^{2+} ion concentration constant and the ATP^{4-} ion concentration at low levels was that it has been reported by Rijksen and Staal (1976b) for human erythrocyte hexokinase that both ions competitively inhibit the enzyme against $MgATP^{2-}$, with inhibition constants of 16 - 18 mM and 1.6 mM respectively. Gerber et al. (1974) found that uncomplexed Mg^{2+} up to 4 mM activated human erythrocyte hexokinase two fold and inhibited it at higher concentrations.

The hexokinase reaction was initiated by the addition of 20 µl of purified hexokinase solution to a 3 ml assay-cuvette. The hexokinase solution had been previously dialysed against the assay buffer (TES-KOH pH = 7.0, I = 0.065) containing 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol for the removal of glucose.

Whereas under saturation of hexokinase with substrates,

the change in absorbance at 340 nm was linear with time at pH = 7.2 and 8.0, an anomalous situation occurred over the pH range of 7.0 to 8.2 with suboptimal concentrations of glucose and $MgATP^{2-}$. The velocity decreased with time during the first ten minutes tested, after the initiation of the hexokinase reaction, and at a greater rate during the first five minutes.

The non-linearity of the reaction decreased by increasing glucose concentrations from 20 to 100 μM at pH = 7 and 8 while $MgATP^{2-}$ concentration had no effect at pH = 7 (Figure 6.4), increased the non-linearity at pH = 8. Non-linearity increased by increasing the pH of the assay solution over the pH range of 7.0 to 8.0 in the presence of 20 μM glucose and 1 mM $MgATP^{2-}$. Additionally the pH optimum shifted to lower pH value (Figure 6.5).

Possible explanations for the decrease of enzyme velocity with time may be thermal inactivation at low levels of enzyme stabilizer, glucose, or proteolytic inactivation of the enzyme although the latter possibility is remote when using material of high purity. Glucose is reported to have a protective effect over heat inactivation for HK I from rat brain (Grossbard and Schimke, 1966), human term placenta (Gustke, 1975) and human erythrocytes (Rijksen and Staal, 1976a) and over proteolytic inactivation of HK I from rat brain (Grossbard and Schimke, 1966).

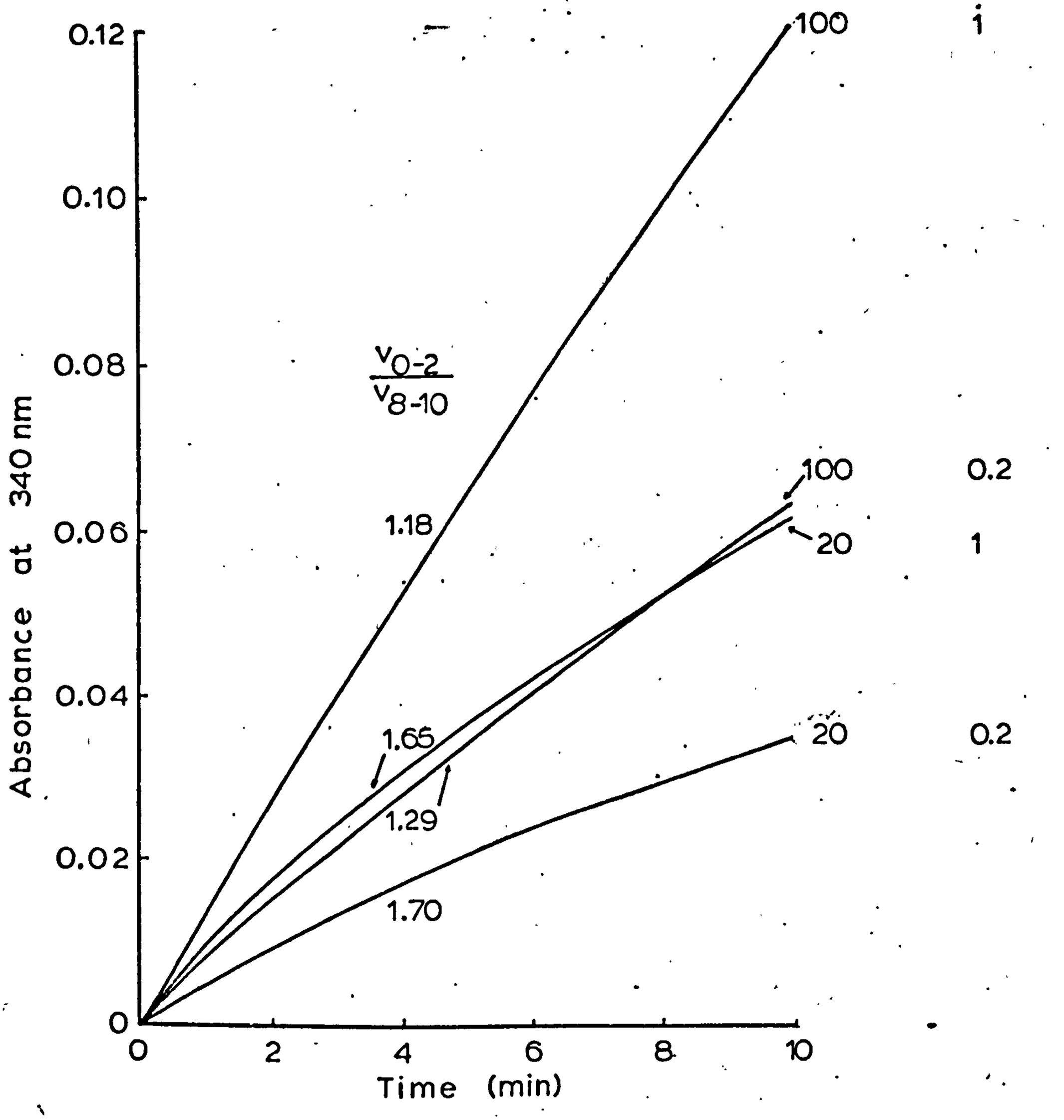


Figure 6.4. Recordings of hexokinase reaction against time at different glucose and $MgATP^{2-}$ concentrations

V_{0-2}/V_{8-10} : Ratio of relative velocities at first two and last two minutes of hexokinase reaction of ten minutes. The assay solution was TES-KOH pH = 7.0, I = 0.065 containing 0.6 mM NADP and 0.5 units/ml G6PD.

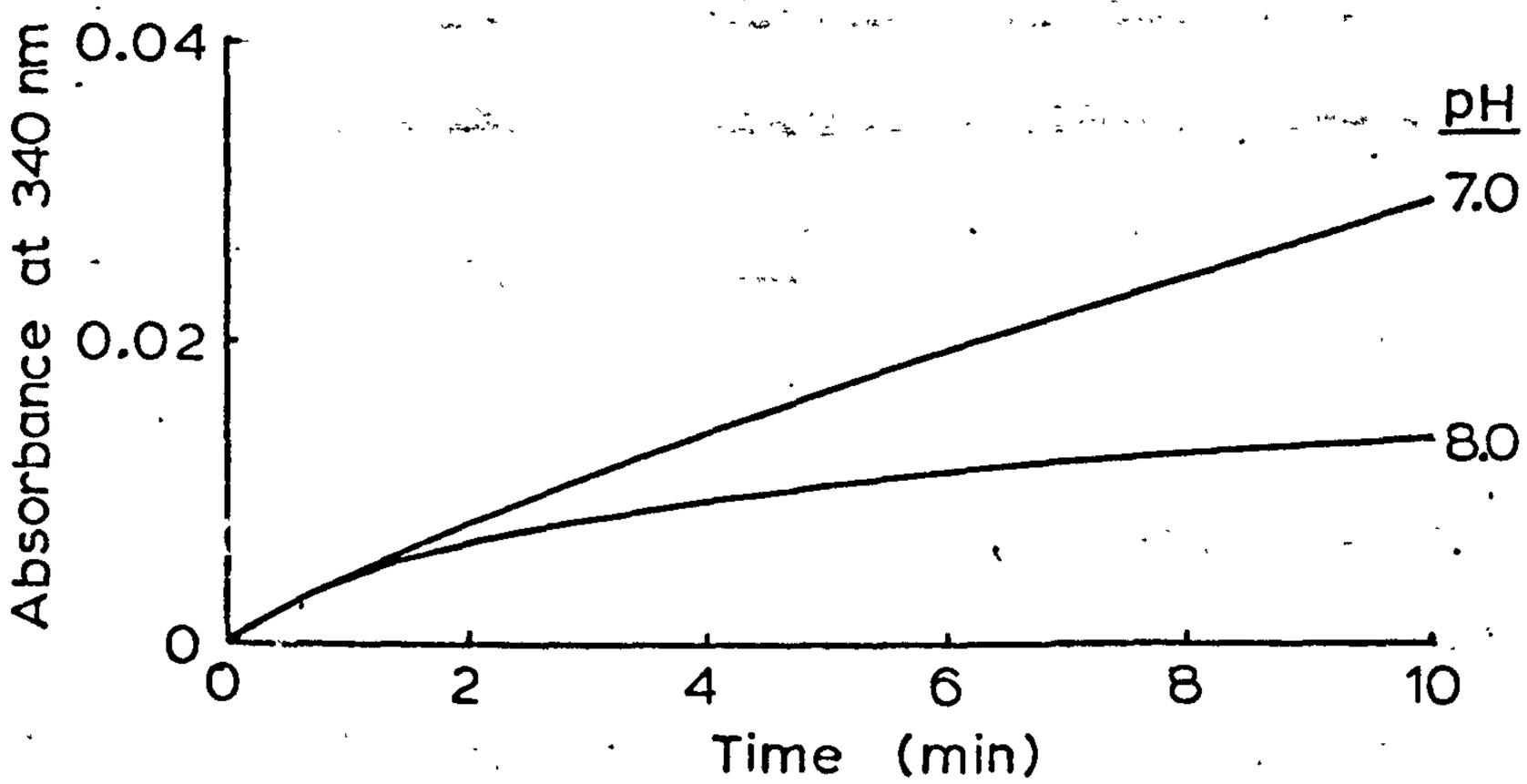
Figure 6.5. Correlation of pH and non-linearity of recording of hexokinase against time

The assay solutions were TES-KOH, I = 0.05 containing 1 mM $MgATP^{2-}$, 20 μM glucose, 0.6 mM NADP and 0.5 units/ml G6PD.

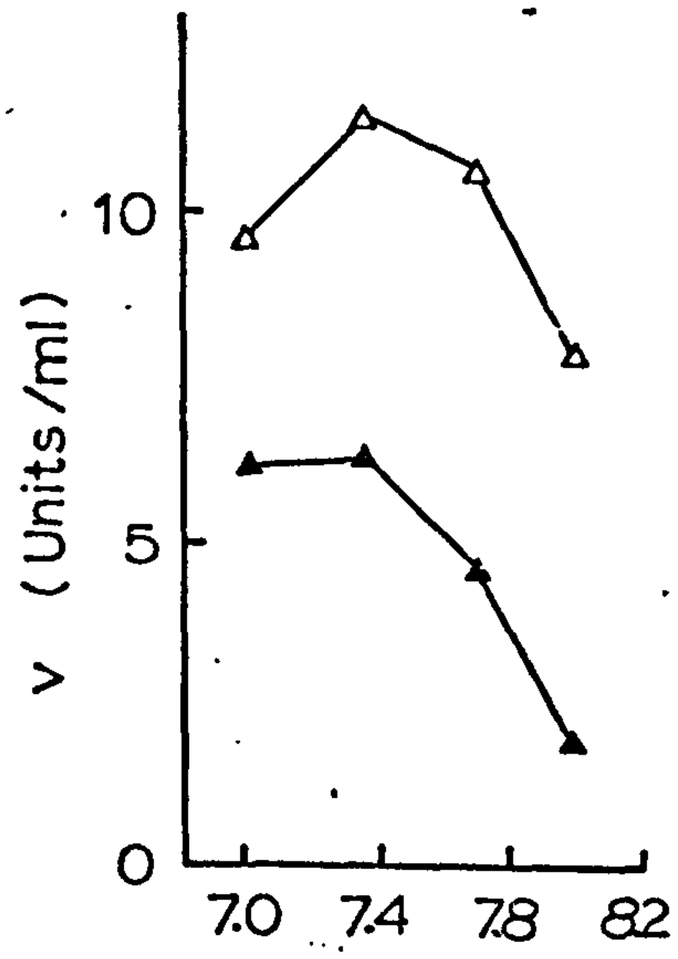
(A) : Change in absorbance at 340 nm during the first 10 minutes of the hexokinase reaction at pH = 7.0 and 8.0.

(B) : Relative velocity at the first two (Δ) and last two (▲) minutes of the hexokinase reaction for ten minutes between pH = 7.0 and 8.0.

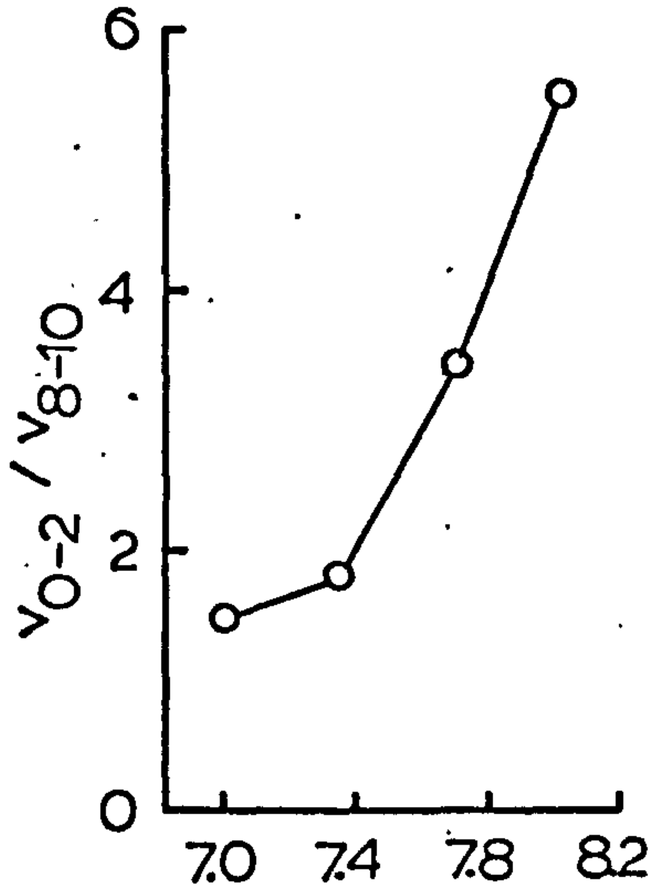
(C) : Ratio of relative velocities at first two and last two minutes of hexokinase reaction of ten minutes between pH = 7.0 and 8.0.


Absorbance at 340 nm
0.04
0.02
0
0
2
4
6
8
10
Time (min)
pH
7.0
8.0

(A)


v (Units/ml)
10
5
0
7.0
7.4
7.8
8.2
pH

(B)


v_{0-2} / v_{8-10}
6
4
2
0
7.0
7.4
7.8
8.2
pH

(C)

For the above reasons pH = 7.0 was chosen for kinetic measurements. Also velocities were calculated from the fifth to tenth minute after the initiation of the reaction because by that time the change in absorbance at 340 nm was approximately linear. However because of the lack of linearity even at 100 μM glucose the results presented below should be considered on a qualitative rather than a quantitative basis.

The calculation of kinetic constants at suboptimal substrate concentration was based on the following equation, according to Alberty (1953).

$$\frac{V}{v} = 1 + \frac{K_A}{[A]} + \frac{K_G}{[G]} + \frac{K_{AG}}{[A][G]}$$

where A and G represent $MgATP^{2-}$ and glucose respectively.

When $MgATP^{2-}$ concentration is varied the ordinate intercept and slope are

$$\text{Intercept} = \frac{1}{V}\left(K_A + \frac{K_{AG}}{[G]}\right)$$

$$\text{Slope} = \frac{1}{V}\left(1 + \frac{K_G}{[G]}\right)$$

Similarly when glucose concentration is varied:

$$\text{Intercept} = \frac{1}{V}\left(K_G + \frac{K_{AG}}{[A]}\right)$$

$$\text{Slope} = \frac{1}{V}\left(1 + \frac{K_A}{[A]}\right)$$

In all cases linear regression with the least squares method was made.

Results are presented in Figures 6.6, 6.7 and 6.8 6.9. Under the assay conditions used, the K_m for glucose was found equal to 51 μM and for $MgATP^{2-}$ equal to 0.32 mM. These values are in accordance with those reported for HK I from rat brain by Grossbard and Schimke (1966), (47 μM and 0.42 mM respectively) and for human erythrocyte hexokinase by Rijksen and Staal (1976a) (64 μM and 0.50 mM respectively). However they differ from those reported by Neumann et al. (1974) (98 μM and 1.4 - 1.6 mM respectively) for the human heart enzyme.

The lines of diagrams 6.6 and 6.8 do not intercept on the abscissa axis. The experiments were done in a buffer of low ionic strength (0.065). At the higher levels of MgATP there is an increase in ionic strength (by 0.02 approximately) which would decrease the activity. An increase in the activity at the higher levels of $MgATP^{2-}$ would shift the data towards a common intercept on

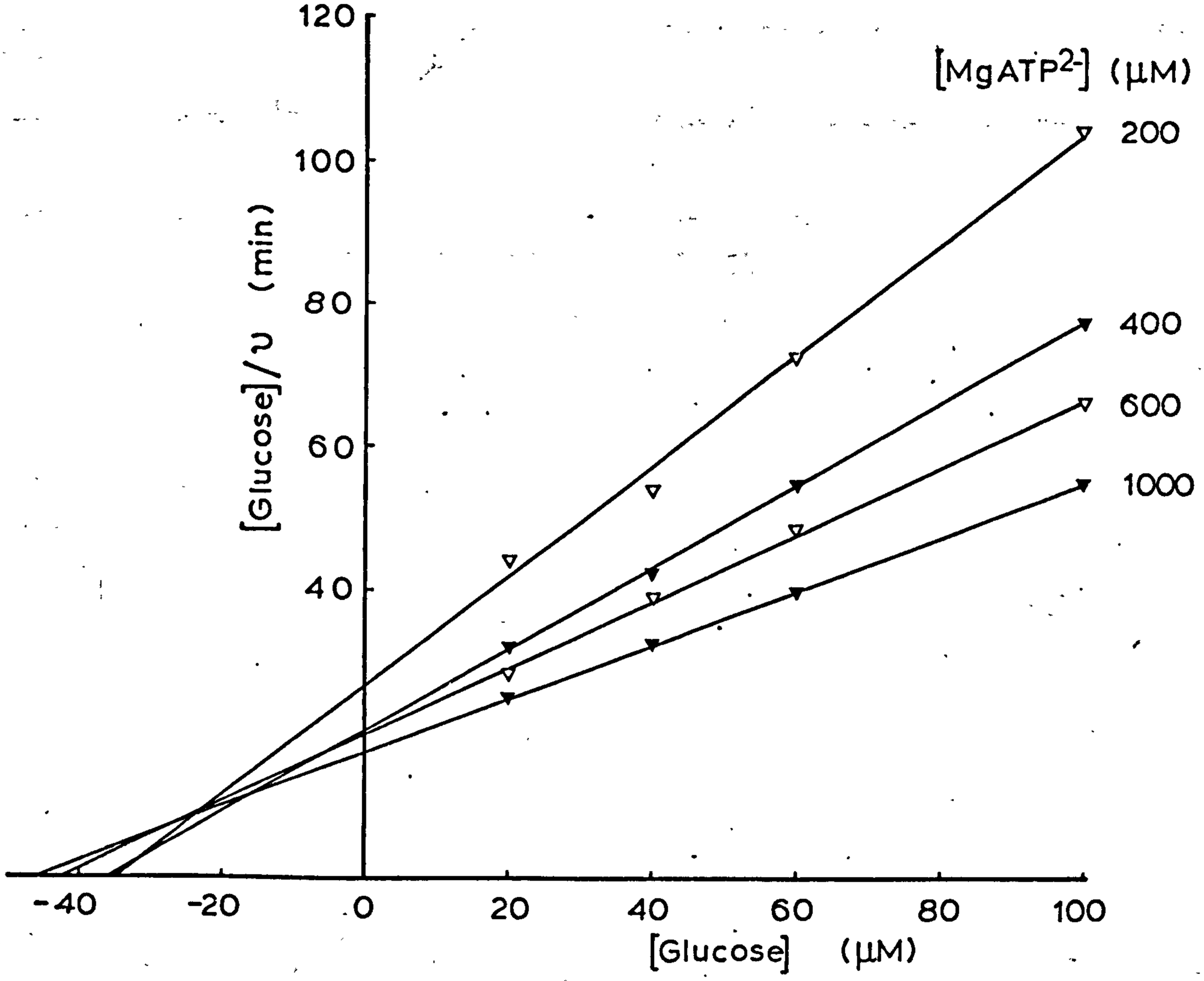
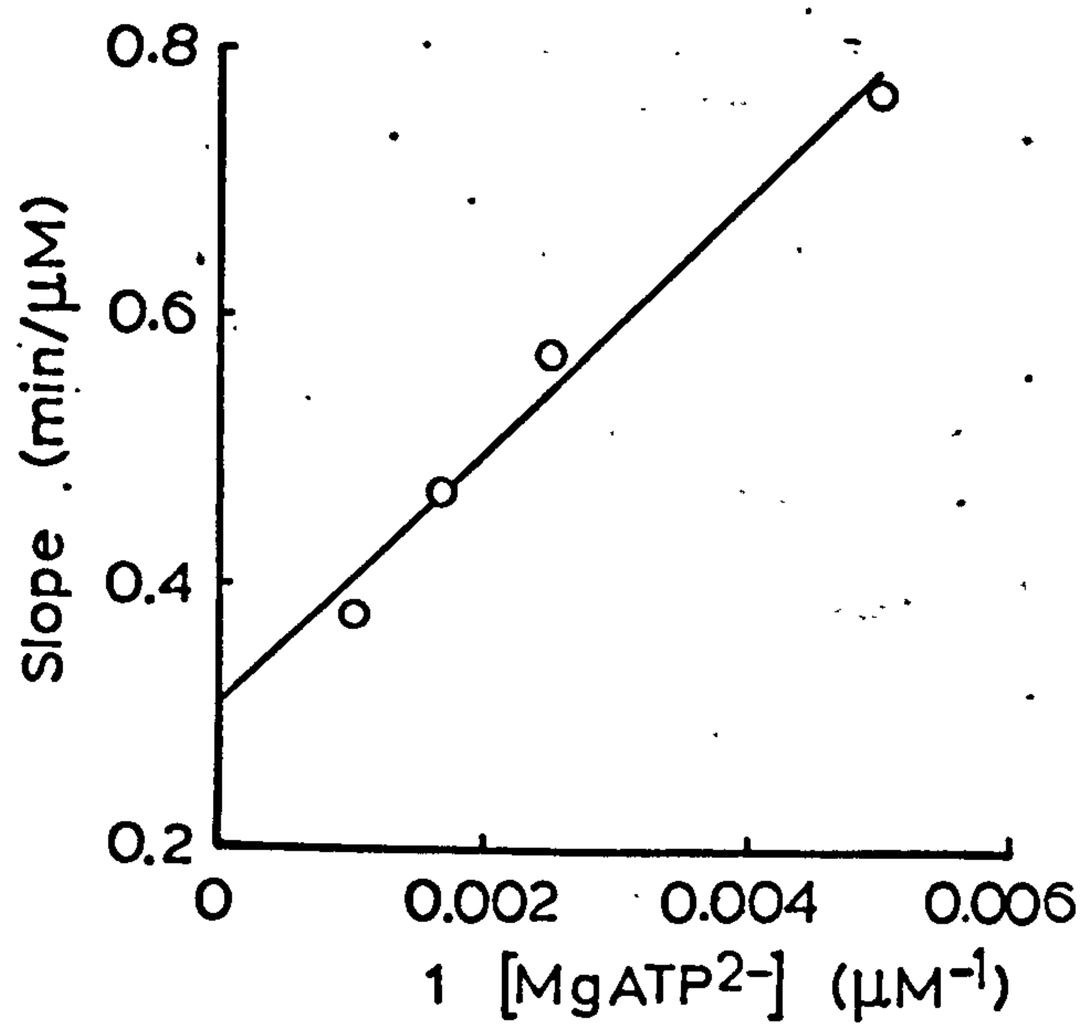


Figure 6.6. Relation between velocity and glucose concentration at 0.2, 0.4, 0.6 and 1.0 mM [$MgATP^{2-}$]

Free [Mg^{2+}] was kept constant at 2 mM.



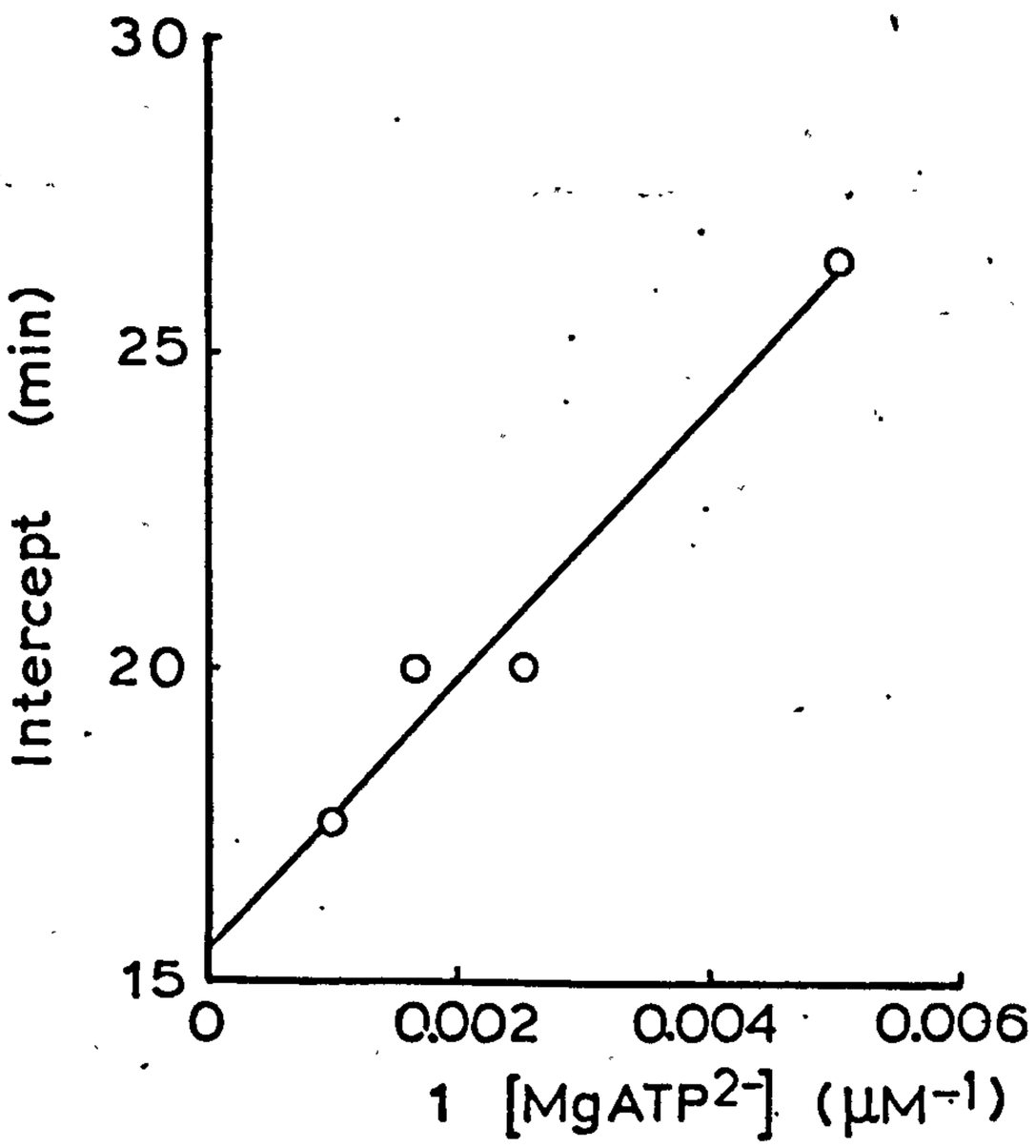


Figure 6.7. Plots of the slope and ordinate intercept versus the reciprocal of $MgATP^{2-}$ concentration from Figure 6.6.

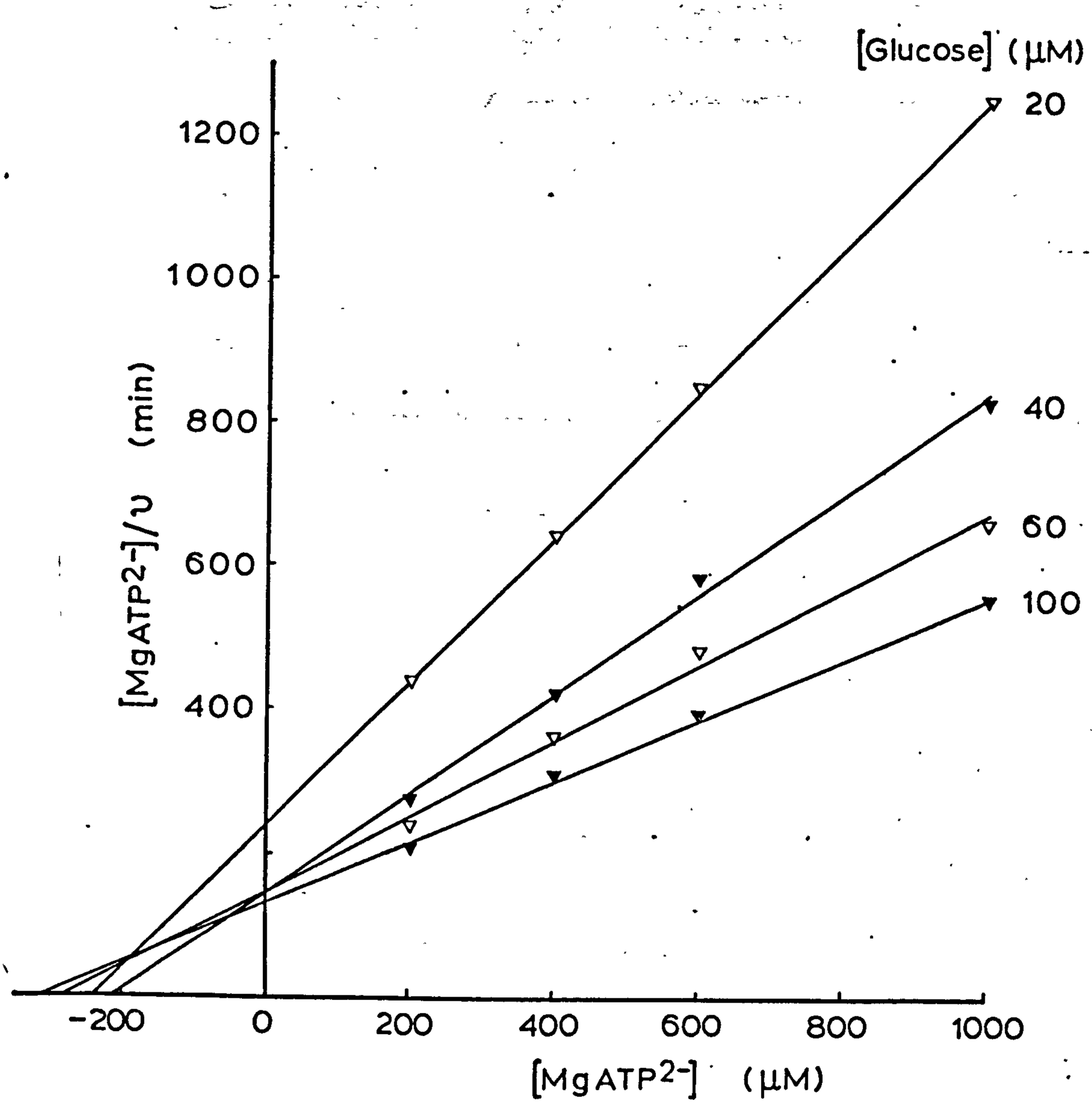
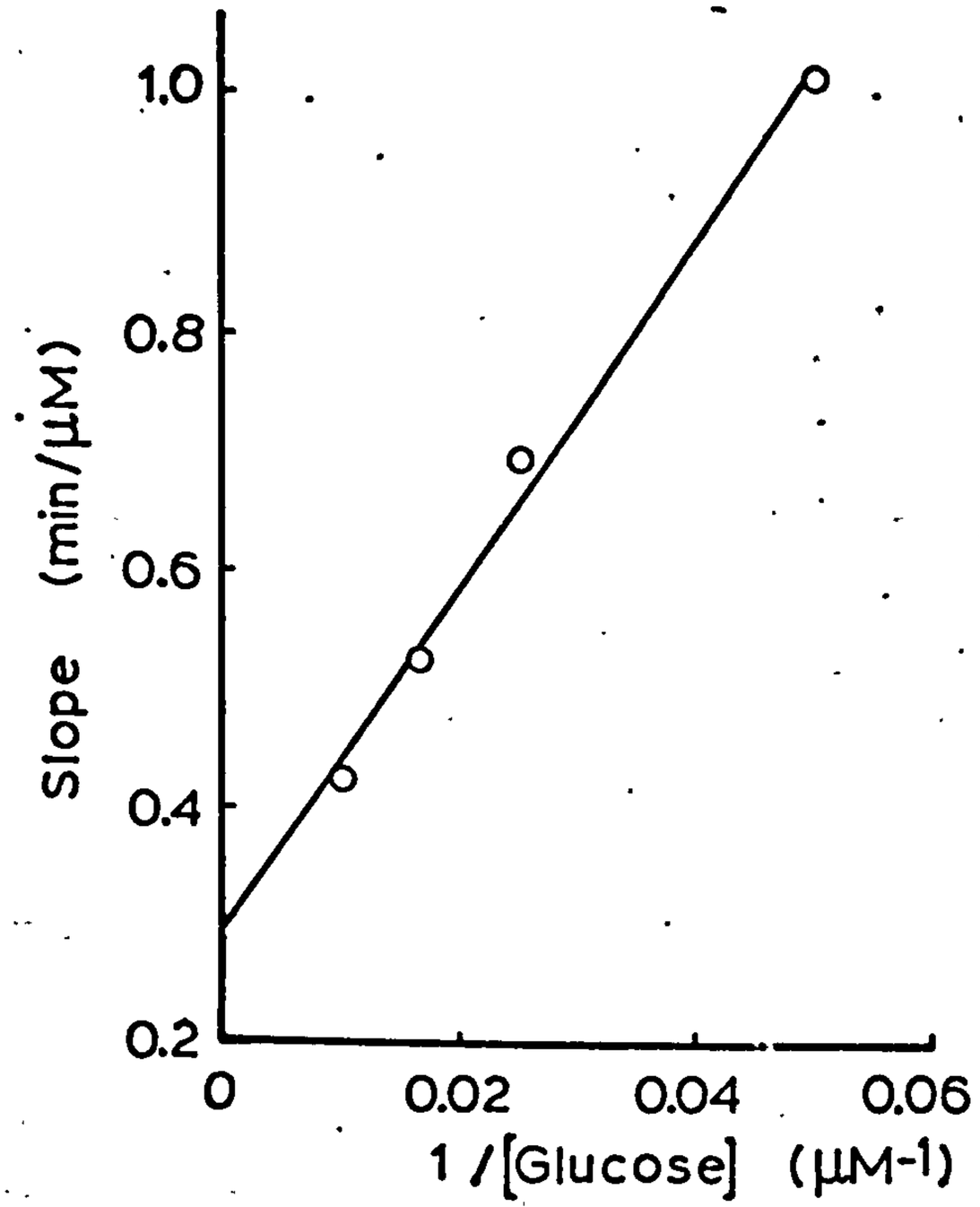


Figure 6.8. Relation between velocity and $MgATP^{2-}$ concentration at 20, 40, 60 and 100 μM [glucose].

Free $[Mg^{2+}]$ was kept constant at 2 mM.



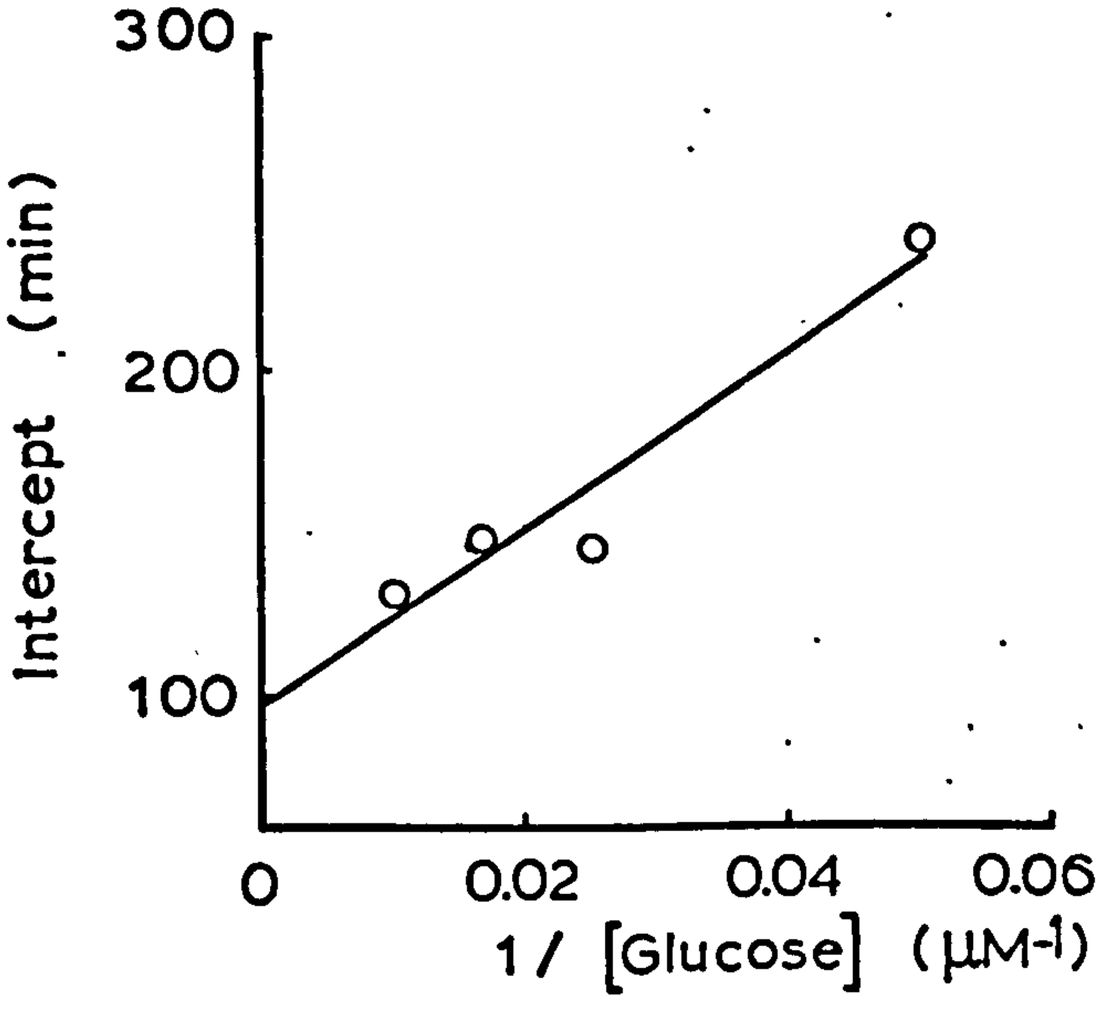


Figure 6.9. Plots of the slope and the ordinate intercept versus the reciprocal of glucose concentration from Figure 6.8.

the x-axis.

By calculation $K_A.K_G$ was found to be greater than K_{AG} by a factor of 2. This means that the two Michaelis constants, K_A and K_G, are not mutually independent. However the non-linearity of the hexokinase reaction does not allow conclusive interpretation of the experimental findings.

A summary of the reported Michaelis constants for glucose and $MgATP^{2-}$ of hexokinase from human tissues and porcine heart is presented in Table 6.2.

Table 6.2. Kinetic constants of hexokinase from human tissues and porcine heart.

Tissue	Km for glucose (μM)	Km for $MgATP^{2-}$ (mM)	Temperature (°C)	Assay conditions	References
Human erythrocytes	64	0.50	25	33 mM Tris-HCl pH = 8.0	Rijksen and Staal (1976a)
Human erythrocytes	38 - 51	1.0-2.0	37	75 mM TES-NaOH pH=7.2 + 75 mM KCl	Gerber et al. (1974)
Human heart (HK I)	98	1.4-1.6	25	74 mM Tris-HCl pH = 7.4	Neumann et al. (1974)
Human spleen (HK III)	30-42	1.4-1.6	25	74 mM Tris-HCl pH = 7.4	Neumann et al. (1974)
Human placenta					
i) solubilized	68	1.44	25	50 mM Tri-ethanolamine-HCl pH = 7.4	Gustke (1975)
ii) soluble	105	1.56	25		
Human gastric mucosa					
i) normal	14-27	—	—	100 mM Tris-HCl pH=8.0	Monakhov et al. (1975)
ii) malignant	18-52	—	—		

cont./

Tissue	Km for glucose (μM)	Km for $MgATP^{2-}$ (mM)	Temperature (°C)	Assay conditions	References
Porcine heart	20	0.44	30	50 mM Tris-HCl pH=7.6	Easterby and O'Brien (1973)
Human heart	51	0.32	25	TES-KOH pH = 7.0 I = 0.065	Present results

SECTION 4

Physical properties

CHAPTER 7

Materials and methods.

(a) Starch gel electrophoresis

Starch gel electrophoresis was performed according to the method of Smithies (1955).

Buffers.

(i) Tris-EDTA-borate buffer pH = 8.6, containing 109 g/l Tris, 5.84 g/l EDTA and 30.9 g/l boric acid (Rosemeyer and Huehns, 1967). This stock buffer was diluted 20 fold for the preparation of gel and 8 fold for the electrode vessels.

(ii) Tris-EDTA-borate-Mg buffered solution. It was prepared by adding 0.025M $MgCl_2$ to buffer (i). This stock buffer was diluted 10 fold for the preparation of gel and 8 fold for the electrode vessels (Rogers et al., 1975b).

(iii) Potassium phosphate buffer pH = 7.0, I = 0.1 containing 2.74 g/l KH_2PO_4 and 4.63 g/l K_2HPO_4. This stock buffer was diluted with water to I = 0.02 for the preparation of gel and 0.06 for the electrode vessels. EDTA was added to both dilutions to a final concentration of

1 mM.

(iv) Potassium phosphate buffer pH = 6.0, I = 0.1 containing 9.75 g/l KH_2PO_4 and 1.65 g/l K_2HPO_4. This stock buffer was diluted with water to I = 0.02 for the preparation of gel and 0.06 for the electrode vessels. EDTA was added to both dilutions to a final concentration of 1 mM.

Preparation of gel.

The gel was prepared by mixing 36 g of hydrolysed starch with 300 ml of the appropriate buffer in a round bottomed flask. The slurry was heated gently until it became viscous and clear. The gel was degassed on a water pump and poured into Perspex trays 22 cm long, 13 cm wide and 0.8 cm deep. The gel was allowed to set at room temperature and finally cooled at $4^{o}C$ for one hour.

Electrophoresis.

The protein samples were soaked into small strips of Whatman No. 3 filter paper (1.3 x 0.5 cm) and inserted into a vertical cut in the gel about 7 cm from one end. Filter paper wicks were used to connect the gel to the electrode vessels. The surface of the gel was covered with a thin sheet of polythene to prevent evaporation. Electrophoresis was carried out at $4^{o}C$. With the Tris-

EDTA-borate system a constant voltage of 480 V was applied for 5 hours while with the Tris-EDTA-borate-Mg system a constant voltage of 200 V was applied for 22 hours. With the potassium phosphate buffer systems a constant current of 50 mA was applied for 5 hours.

Stain for hexokinase activity and protein. Preservation of gels

After electrophoresis, gels were sliced into three horizontal sections. The thin middle section was stained for protein and the lower cut surface was stained for enzyme activity.

Hexokinase activity was developed by pouring 20 ml of the staining solution described for polyacrylamide gels and incubating the system at room temperature for about $\frac{1}{2}$ hour in the dark.

For the staining of proteins the gels were overlaid with a 1% (w/v) solution of naphthalene black in methanol-acetic acid-water (5:1.4, v/v/v). The stain was allowed to soak into the gel for about 5 minutes and the background stain removed by repeated washings with the same solvent for 1-2 days.

The gels were preserved in methanol-acetic acid-water (5:1.4, v/v/v).

(b) Cellulose acetate electrophoresis

Buffers.

(i) Tris-citrate buffer pH = 8.6, containing 9.2 g/l Tris and 1.05 g/l citric acid.

(ii) Borate-NaOH buffer pH = 8.6, containing 92.75 g/l boric acid and 0.3 moles/l NaOH.

Electrophoresis

Sepharose III cellulose polyacetate electrophoresis strips (15.2 cm long and 2.5 cm wide) were impregnated with Tris-citrate buffer pH = 8.6 by floating them onto the surface of the buffer and subsequently immersing them for at least 10 minutes. The strips were blotted between 2 pieces of blotting paper avoiding excess drying (indicated by the appearance of white spots) and placed on the shoulder pieces of a Shandon Cellulose Acetate Electrophoresis tank. For wicks double thickness of Whatman paper No 1 was used. The electrode vessels contained borate-NaOH buffer pH = 8.6. A small quantity of protein sample (5-10 µl) was applied with a micropipette as an even streak about 2 cm from the cathode wick. The electrophoresis was completed in 50 minutes at a constant voltage of 250 V.

Stain for hexokinase activity and protein. Preservation of strips.

Hexokinase activity was visualised by immersion in the same staining solution described for polyacrylamide gels, for 5 minutes at room temperature in the dark.

For the protein stain, the strips were immersed in a 1% (w/v) solution of naphthalene black in methanol-acetic acid-water (5:1:4, v/v/v) for 5 minutes. The background stain was rapidly removed by repeated washings in the same solvent.

The developed strips were preserved in a methanol-acetic acid-water (5:1:4, v/v/v) solution. A gradual fading of formazan bands occurred with time.

(c) Polyacrylamide gel electrophoresis

Disc electrophoresis was performed in 5 and 7.5% polyacrylamide gels using modifications of the methods of Ornstein (1964) and Davies (1964).

Buffers.

(i) Tris-HCl buffer pH = 6.7, containing 0.45 moles/l HCl and 50 g/l Tris, titrated to pH = 6.7 with additional Tris.

(ii) Tris-HCl buffer pH = 8.9, containing 0.48 moles/l HCl and 366 g/l Tris, titrated to pH = 8.9 with additional Tris.

(iii) Tris-glycine buffer pH = 8.5, containing 3 g/l Tris and 14.4 g/l glycine, titrated with additional Tris.

(iv) Acrylamide solution containing 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide.

(v) Sample buffer containing 2 ml glycerol, 0.5 ml 0.1% (w/v) bromophenol blue, 0.1 ml Tris-HCl buffer pH = 6.7 (buffer (i)) and 7.5 ml water.

Preparation of gel.

The gel solution was prepared by adding 4.2 or 6.2 ml of acrylamide solution to give a final concentration of acrylamide of 5 and 7.5% (w/v) respectively, 3.1 ml Tris-HCl buffer pH = 8.9, 10 µl of Tetraethylene methylene diamine (TEMED) and water up to the volume of 25 ml. The solution was degassed in an aspirator connected on a water pump. Polymerization was initiated by the addition of 50 or 250 µl of a 10% (w/v) freshly prepared ammonium persulphate solution in water, depending on whether a slow (within 30 minutes) or a fast (within 8 minutes) polymerisation was required. The fast polymerisation was preferred when G6PD was incorporated in the gel solution because

it was shown that this enzyme is rapidly inactivated by the acrylamide and bisacrylamide monomers (Harrison, 1974). The concentration of G6PD in the gel solution was 0.56 units/ml. Immediately after the addition of ammonium persulphate the gel solution was poured into glass tubes 7.5 x 0.5 cm and overlaid with water.

Electrophoresis.

After the completion of polymerization, the tubes were inserted into a Shandon disc electrophoresis assembly and the electrode compartments filled with Tris-glycine buffer pH = 8.5. A small volume, not more than 50 µl, of protein solution was applied with a micropipette on the tubes. This protein solution was prepared by mixing equal volumes of the protein sample and the sample buffer. Electrophoresis was carried out at $4^{o}C$ with a constant current of 2 mA per tube. It was completed when the bromophenol blue marker band reached 0.5 cm from the anode end of the tubes.

Stain for hexokinase activity.

Each gel was incubated in a test tube containing the following staining solution, at room temperature and in the dark.

Tris-HCl buffer pH = 8.0, I = 0.05 containing

5 mM ATP

10 mM $MgCl_2$

100 mM glucose

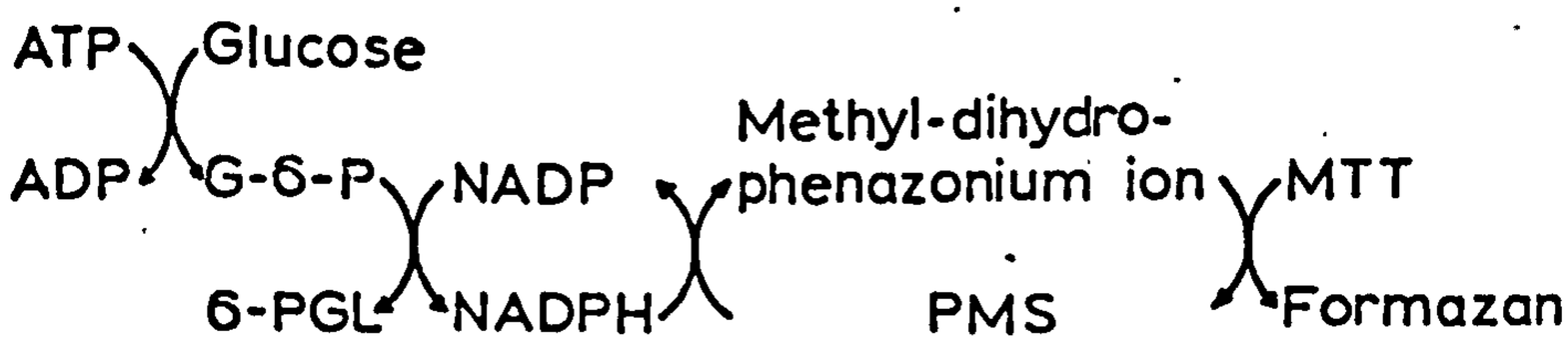
0.6 mM NADP

0.5 units/ml G6PD

0.5 mg/ml PMS

0.5 mg/ml MTT

If G6PD was incorporated in the gel, staining was performed as above except that this enzyme was omitted from the staining solution. Brown bands were developed in the areas of hexokinase activity due to the formation of insoluble formazan by the following set of reactions.



Protein stain.

The gels were stained for protein by incubation in a 0.2% (w/v) solution of Coomassie Brilliant Blue R in methanol-acetic acid-water (25:7:68, v/v/v) for 12 hours.

The background stain was removed by repeated washing in the same solvent. Gels stained for protein did not contain G6PD since this enzyme produced a background protein stain.

Preservation of gels.

Gels stained for hexokinase activity and for protein were kept in methanol-acetic acid-water (25:7:68, v/v/v) solution. However hexokinase activity bands faded with time.

(d) SDS polyacrylamide electrophoresis

One of the most critical tests of protein homogeneity is the polyacrylamide gel electrophoresis in the presence of the anionic detergent, sodium dodecyl sulphate (SDS). This method is normally used for determining the subunit composition of a protein, but by loading a large protein sample onto the gel the presence of small amounts of impurities may be detected. It has been also shown that the separation of proteins by SDS electrophoresis is dependent on the molecular weight of their polypeptide chains (Shapiro et al., 1967) so that when the electrophoretic mobilities of polypeptide chains were plotted against the logarithms of their molecular weights a smooth curve was obtained. Certain factors have been found to produce artifacts, e.g. lipid and carbohydrate attached to pro-

teins, or incomplete binding of SDS to proteins.

SDS polyacrylamide gel electrophoresing was performed using a system based on the methods of Weber and Osborne (1969) and Laemmli (1970) using 5 and 10% (w/v) polyacrylamide gels.

Buffers.

They were the same as for the normal polyacrylamide gel electrophoresis with the following modifications:

i) The electrode buffer, Tris-glycine pH = 8.5 contained also 0.1% (w/v) SDS.

ii) The sample buffer contained also 2.5% (w/v) SDS and 2% (v/v) 2-mercaptoethanol.

Preparation of gel.

The gel solution was prepared by adding 4.2 or 8.4 ml of acrylamide solution to give a final concentration of acrylamide monomer 5 and 10% (w/v) respectively, 3.1 ml Tris-HCl buffer pH = 8.9, 0.5 ml of a 5% (w/v) SDS solution in water, 10 µl TEMED, and water up to a volume of 25 ml. The gel solution was degassed before the addition of SDS, and polymerization was initiated by the addition of 50 µl of a freshly prepared 10% (w/v) solution of ammonium persulphate in water. The gels were poured in glass tubes 12 x 0.5 cm and overlaid with a small volume

of 0.1% (w/v) solution of SDS in water. After 30 minutes polymerization was complete.

Preparation of protein samples and electrophoresis.

Equal volumes of protein solution and sample buffer were mixed in narrow tubes (2 mm diameter) and boiled in a water bath for 5 minutes to reduce the protein fully.

The surface of the gels was flushed with electrode buffer and the tubes assembled in a Shandon disc electrophoretic apparatus. Up to 20 µl of reduced protein sample was applied to the surface of the gel. Electrophoresis was carried out at room temperature with a constant current of 2 mA per tube until the bromophenol blue marker band reached 1 cm from the anode end of the tubes.

Staining of gels for proteins, glyco and lipoproteins. Preservation of gels.

After electrophoresis the gels were immersed in a 15% (w/v) solution of trichloroacetic acid in water for 2 hours to precipitate the proteins.

(i) General protein stain

Gels were stained with Coomassie brilliant blue R as described for normal polyacrylamide gels. The absorption profile of the gels at 265 nm was recorded with a Joyce Loebl u.v. scanner connected to a Bryans 27000 chart

recorder.

(ii) Stain for glycoproteins

For the detection of glycoproteins, gels were stained according to the method of Zacharius et al., (1969). The gels were rinsed lightly with water and were immersed in a 1% (w/v) solution of periodic acid in 3% (v/v) acetic acid in water for 50 minutes. They were thoroughly washed with several changes of water and shaking for 24 hours for the complete removal of IO_3^- ions. The gels were transferred to test tubes containing the fuchsin-sulphite stain prepared as described below and kept in the dark for 50 minutes. Afterwards the gels were washed with freshly prepared 0.5% (w/v) metabisulphite solution in water.

The fuchsin-sulphite stain was prepared as follows:

1 g of basic fuchsin was dissolved in 100 ml of boiling water. The solution was cooled to 60°C and 2 g of potassium metabisulphite was added. Subsequently 20 ml of 1N HCl solution was added slowly. The solution was left overnight in a stoppered flask. The solution was decolourized with charcoal and filtered.

(iii) Stain for lipoproteins

For the detection of lipoproteins, gels were stained according to the method of Craven and Basford (1974). The

gels were immersed in a Sudan black B solution and left overnight. The sudan black B solution was prepared by mixing 10 ml of a saturated solution of sudan black B in ethanol, 4 ml glycerol, 6 ml water and 1 ml acetic acid. Background stain was removed by soaking in a glycerol-water-acetic acid solution (4:16:1, v/v/v).

(e) Analytical gel filtration

Analytical gel filtration can be used as an indication of size homogeneity and for initial estimation of molecular weight, diffusion coefficient and Stokes' radius.

(i) As an indication of homogeneity

By heavily loading a long Sephadex G-200 column with a protein sample and studying the elution profile, an indication of the solute homogeneity with respect to size can be obtained if the enzyme activity peak coincides with the protein peak and the specific activity is constant throughout it. However neither the resolution nor the sensitivity of absorbance at 280 nm are clear indications of homogeneity.

(ii) Initial estimation of molecular weight

Gel filtration on Sephadex G-200 was used by Andrews (1964, 1965) as a comparative method giving estimates of

the molecular weight of proteins. The elution volume of the protein was related to the logarithm of molecular weight. The relationship was found to hold for many of the proteins studied, although certain exceptions were noted, especially for those proteins with a highly asymmetrical shape, or those with a high proportion of carbohydrate.

(iii) Initial estimation of diffusion coefficient and Stoke's radius

The elution of proteins through dextran polymers may be considered as a function of the Stoke's radius of the molecule (Ackers, 1964; Siegel and Monty, 1966). The Stoke's radius may be related to the diffusion coefficient of a molecule using the Stoke's-Einstein law for the free diffusion of spherical particles $D = \frac{kT}{6\pi\eta r}$. Thus the elution of a protein may be related to its' diffusion coefficient. Experimentally a smooth relationship was found to hold for many proteins when their elution volume was plotted against the reciprocal of diffusion coefficients (Andrews, 1965).

(iv) Conditions for analytical gel filtration

Sephadex G-200 columns were poured as described in Chapter 2. For the purpose of checking homogeneity the column had dimensions of 150 x 2 cm and the flow rate was 8 ml/hour. For estimation of molecular weight and diffusion coefficient, the column had dimensions 140 x 1 cm and

the flow rate was 7 ml/hour. Samples with the markers and hexokinase were run separately. Both types of gel-filtration experiments were performed at $4^{o}C$. Phosphate buffer pH = 7.0, I = 0.1 containing 10 mM glucose, 1 mM K_2EDTA and 0.1% (v/v) 2-mercaptoethanol was used both for dialysis of the samples chromatographed and for their elution from the columns.

(f) Sedimentation velocity

Sedimentation velocity experiments were performed using a Beckman Spinco model E, analytical ultracentrifuge, equipped with an RTIC unit. All experiments were carried out at $10^{o}C$. A standard A -D rotor (maximum speed 59,780 r.p.m.) was used and the radial co-ordinates defined by the counterbalance reference edges. Ilford rapid orthometallic plates were used with the schlieren optical system and the plates measured with a twin coordinate travelling microscope.

All sedimentation velocity experiments were performed at 59,780 r.p.m. Standard cells fitted with sapphire windows and 12 mm Kel.F centerpieces of 4^{o} sector angle were routinely used. The cells were filled with 0.6 - 0.7 ml of protein solution, normally about 5 mg/ml.

Sedimentation coefficients were measured by taking

photographs at equal time intervals, after the schlieren peak had spearated from the meniscus.

The sedimentation coefficient is defined by the equation $s = \frac{1}{w^2 r} \cdot \frac{dr}{dt}$ and the integrated form was used to obtain s

$$\log_{10} r = \frac{sw^2 t}{2.303} + \text{constant}$$

where s is the sedimentation coefficient

r is the radial distance

w is the rotor speed in radians/s

t is the time interval between measured position of r

By plotting $\log_{10} r$ against t, a straight line should be obtained for a system of a single discrete protein entity, the slope of which is proportional to s. A least mean squares procedure was used to determine the slope of the graph.

The observed sedimentation coefficient obtained for the temperature and solvent conditions of the centrifugation was corrected to the standard $s_{20.w}$ values using the equation

$$s_{20.w} = s_{obs} \left(\frac{\eta_t}{\eta_{20}}\right)_w \cdot \left(\frac{\eta_b}{\eta_w}\right)_t \cdot \frac{(1-\bar{v}\rho)_{20.w}}{(1-\bar{v}\rho)_{t.b}}$$

where $\eta_{b.t}$ is the viscosity of buffer at temperature t

$\rho_{w.20}$ is the density of water at 20°C

$\bar{v}$ is the partial specific volume of the protein

The viscosities and densities of solvents were taken from International Critical Tables. The ionic strength was generally 0.1 or greater to minimize charge effects.

(g) Boundary formation and the measurement of diffusion coefficient

For the determination of protein concentrations, artificial boundaries between solvent and protein solution were formed using a double sector, synthetic boundary centerpiece. Two scribe lines at the top and middle of one face of the centerpiece allowed passage of solvent from one sector to the other, with the concurrent displacement of air. 0.15 ml of the protein solution was placed in one sector and 0.45 ml of the corresponding buffer, against which the protein solution had been dialysed, was placed in the other sector. The rotor speed was gradually increased and the boundary seen to form at 1,000 rpm until the excess buffer had layered on top of the protein solution. The rotor was stabilized at 6,995 rpm. At this speed it was thought that negligible sedimentation would occur during the diffusion experiments.

For the boundary forming experiments, two sets of photographs were taken at various phase plate angles, allowing the boundary to diffuse between each set. For the diffusion experiment photographs were taken at a constant time interval and fixed phase plate angle.

The protein concentration was estimated from the boundary forming experiment by trapezoidal integration of the schlieren peak.

The diffusion coefficient was calculated from the equation

$$D = \frac{1}{4\pi t}\left(\frac{A}{H}\right)^2 .(1 - w^2 st). \qquad \text{(Schachman, 1959)}$$

where A is the peak area, calculated by trapezoidal integration

H is the peak's height

t is the time interval from the start of the experiment.

At the low speed employed for the diffusion experiments the term $w^2 st$ is negligible.

Values of $(A/H)^2/2\pi$ were plotted against t and the slope of the line calculated by the least mean squares method.

The observed diffusion coefficient for the protein at

the temperature and solvent conditions of the experiment was corrected to a $D_{20.w}$ value using the equation

$$D_{20.w} = D_{obs.} \cdot \frac{293}{T} \cdot \left(\frac{\eta t}{\eta_{20}}\right)_w \cdot \left(\frac{\eta b}{\eta_w}\right)_t$$

where T is the absolute temperature

$\eta_{b.t}$ is the viscosity of buffer at temperature t

$\eta_{w.20}$ is the viscosity of water at $20^{o}C$.

(h) Sedimentation equilibrium

Sedimentation equilibrium experiments were performed according to the method of Van Holde and Baldwin (1958), using Schlieren optics and solution columns of 3 mm. Cells fitted with Epon-Aluminium double sector centerpieces (12 mm, 2^{o} sector angle) and sapphire windows were used. One sector was loaded with 0.11 ml of protein solution, the other with 0.13 ml of corresponding buffer.

The speed of centrifugation was selected to give a value of 0.9 for α, defined as $\alpha = \frac{RT}{M_r\omega^2(1-\bar{v}\rho)} \cdot \frac{2}{(b^2-a^2)}$ where a and b are the radial positions at the top and bottom of the protein solution. This value of α is equivalent to selecting a speed to obtain a value of 3 for the ratio C_b/C_a where C_a and C_b are the protein concentrations at the top and bottom of the protein solution.

The minimum length of time for the equilibrium experiment was determined using the equation:

$$t = \frac{(b - a)^2}{D} \cdot F(\alpha)$$

where $F(\alpha)$ is a complex function of α and of the difference in protein concentration between the top and the bottom of the solution column. This equation defines a condition in which $(C_b - C_a)$ is within 0.1% of the concentration difference expected at equilibrium. (This error is much less than the limits of resolution of the measurements). $F(\alpha)$ converges rapidly to a value of 0.68 for values of $\alpha > 0.6$.

The basic flow equation in the sedimentation equilibrium experiments derived by substituting the Svedburg equation in the Lamm (1929) equation.is

$$\frac{1}{rc} \cdot \frac{dc}{dr} = 2AM \qquad \text{(Equation 1)}$$

where $$A = \frac{\omega^2(1-\bar{\upsilon}\rho)}{2RT}$$

c is the protein concentration

r is the distance from the centre of rotation of the centrifuge rotor

$\frac{dc}{dr}$ is the corresponding concentration gradient

M is the molecular weight

The initial concentration C_o was obtained from boundary forming experiments. The meniscal concentration C_a was obtained by double integration:

$$\int_a^b (r \int_a^r \frac{dc}{dr} \cdot dr) dr = (c_o - c_a) \cdot \frac{(b^2 - a^2)}{2}$$

The mean value of the weight average molecular weight across the cell, Mw, was calculated by the following equation:

$$M_w = \frac{(c_b - c_a)}{c_o} \cdot \frac{1}{A(b^2 - a2)}$$

The mean value for the "Z average" molecular weight across the solution column, Mz, was calculated from the following equation:

$$M_z = \frac{\left(\frac{1}{r}\frac{dc}{dr}\right)_b - \left(\frac{1}{r}\frac{dc}{dr}\right)_a}{c_b - c_a} \cdot \frac{1}{2A}$$

Heterogeneity of preparations was detected by:

(i) the ratio $\frac{Mz}{Mw}$ which is unity when one molecular species is present.

(ii) From the integrated form of equation 1,

$\ln c_r = \ln c_a + AM(r^2 - a2)$, a plot of $\ln c_r$ versus r^2 should give a straight line for homogeneous material. The slope of this line would be $\overline{Mw}$

(iii) From equation 1, a plot of $\frac{1}{r}.\frac{dc}{dr}$ versus $(C_r - C_a)$ should give a straight line for homogeneous material. The slope of this line would be $\overline{Mz}$.

(iv) From equation 1, the molecular weight at various points in the solution is $Mw(r) = \frac{1}{2A} \cdot \frac{1}{rc} \cdot \frac{dc}{dr}$. This value should be constant throughout the solution column for homogeneous material. The values may be summed across the column to give a mean value $\overline{Mw}(r)$.

(i) <u>Sucrose gradient ultracentrifugation</u>

Sucrose gradient preparative ultracentrifugation was performed according to the method of Martin and Ames (1961) for the estimation of the sedimentation coefficient of hexokinase by interpolation between markers of known values of $s_{20.w}$.

A Beckman L2-65B preparative ultracentrifuge with a swinging bucket rotor (SW40 Ti) was used. Polycarbonate tubes (9.5 x 1.4 cm) of 14 ml capacity were slowly filled from the outlet of a gradient mixer. The mixer consisted of two equal cylindrical chambers built in a block of perspex glass, interconnected at the bottom with a stop-cock. One chamber contained a 20% (w/v) sucrose solution in phosphate buffer pH = 7.0, I = 0.1 plus 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. The other

contained a 5% (w/v) sucrose solution in the same buffer. The latter chamber served also for the mixing of the gradient with the help of a spinning platinum loop. Polythene tubing was attached with the bottom of this chamber with a glass micropipette on its' other end. The outflow of the gradient to the bottom of the tubes was adjusted with a screw clamp. In this way the gradient was inverted in the tubes and the final sucrose concentration at the top and bottom of the tubes was 5 and 20% respectively. The tubes were left for some hours to stand at $4^{o}C$ before the application of 0.5 ml samples containing hexokinase and the markers previously dialysed against the above buffer. The samples were layered at the top of the gradient with a micropipette and the tubes were centrifuged for 16 hours at 40,000 r.p.m. at $4^{o}C$. Fractions were collected by fixing each tube in a cylindrical chamber having two outlets at its' bottom and top. A hole was punched to the tube from the bottom outlet of the chamber and 50% (w/v) sucrose solution in the buffer was pumped in with a peristaltic pump. The gradient was thus forced upwards and flowed out from the top of the chamber. Equal fractions of 16 drops (0.5 ml) were collected and analysed.

CHAPTER 8

Erythrocyte hexokinase

(a) Electrophoresis

Electrophoretic studies of adult human erythrocyte hexokinase isoenzymes are conflicting showing, on starch gels, 7 bands (Eaton et al., 1966), or HK I and HK III (Holmes et al., 1967; Holmes et al., 1968; Schröter and Tillman, 1968; Neumann et al., 1974), or HK I, HK II and HK III (Malone et al., 1968), or a double HK I band and HK III (Kaplan and Beutler, 1968), or a triple HK I band and HK III (Rijksen et al., 1977), or double bands for both HK I and HK II (Rogers et al., 1975b). On agarose gel electrophoresis 4 bands were observed (Altay et al., 1970).

A common feature is the persistence of HK I in all investigation mentioned above. Also in almost all cases HK I band(s) represented most of the enzyme activity of the samples. Inconsistencies in number and isoenzyme types presumably arise from differences in experimental conditions and criteria for characterizing isoenzymes.

(1) Starch gel electrophoresis

The purified erythrocyte hexokinase was electrophor-

esed on starch gel under conditions described in Chapter 7. With the Tris-EDTA-borate buffer pH = 8.6, after a 5 hour electrophoresis, a single anodal band was stained for activity with mobility slightly slower than that of CO-haemoglobin used as marker. However after electrophoresing with the Tris-EDTA-borate-Mg buffer, pH = 8.6, for 22 hours at lower voltage, four hexokinase bands of almost equal staining intensity were revealed with relative mobilities to haemoglobin equal to 0.9, 0.75, 0.65 and 0.53. These values were almost identical to those found by Rogers et al. (1975b) for fresh haemolysates under the same electrophoretic conditions. It is difficult to explain the different patterns on electrophoresis in Tris-EDTA-borate and Tris-EDTA-borate-Mg buffers. The additional bands could arise from variation in bound ions, or possibly different conformations of the enzyme. According to Rogers et al. (1975b) the multi-banded pattern of low Km hexokinases in several human tissues may have arisen as a result of secondary modifications of the "primary" isoenzyme, where the "primary" isoenzyme is usually the least anodal band of a multi-banded complex.

With the phosphate buffer solution of pH = 7.0 one band of activity was revealed throughout the purification procedure moving 1.2 cm towards the anode, whereas

CO-haemoglobin moved 0.45 cm towards the cathode.

The isoelectric point for erythrocyte hexokinase was found as 6, as enzyme activity was observed only in the origin after electrophoresis with phosphate buffer system pH = 6.0. Easterby and O'Brien (1973) reported the same value for porcine heart hexokinase by the same method. The isoelectric point of rat brain hexokinase was found to be between 6 and 6.5 by isoelectric focussing (Chou and Wilson, 1972). Rijksen and Staal (1976a) by isoelectric focussing of the purified human erythrocyte enzyme found a major protein band with the rather low value of pH = 4.7. However the band shown may represent a major impurity of their preparation.

(2) Polyacrylamide gel electrophoresis

Electrophoresis of the purified erythrocyte hexokinase with 5 and 7.5% normal polyacrylamide gels (as described in Chapter 7) with a protein loading of 2 µg per tube revealed one band of hexokinase activity, stained with G6PD either in the staining solution or in the gel. By increasing the protein loading to 50 µg per tube, one major and one faster minor protein band was revealed after staining for protein or scanned at 265 nm (Figure 8.1). Hexokinase activity was still represented by one band coinciding to the central area of the major protein band.

Incubating the hexokinase sample for 24 hours in Tris-HCl buffer pH = 8.0, I = 0.01 plus 0.1 M KCl, 1 mM glucose 6-phosphate, 0.1% (v/v) 2-mercaptoethanol

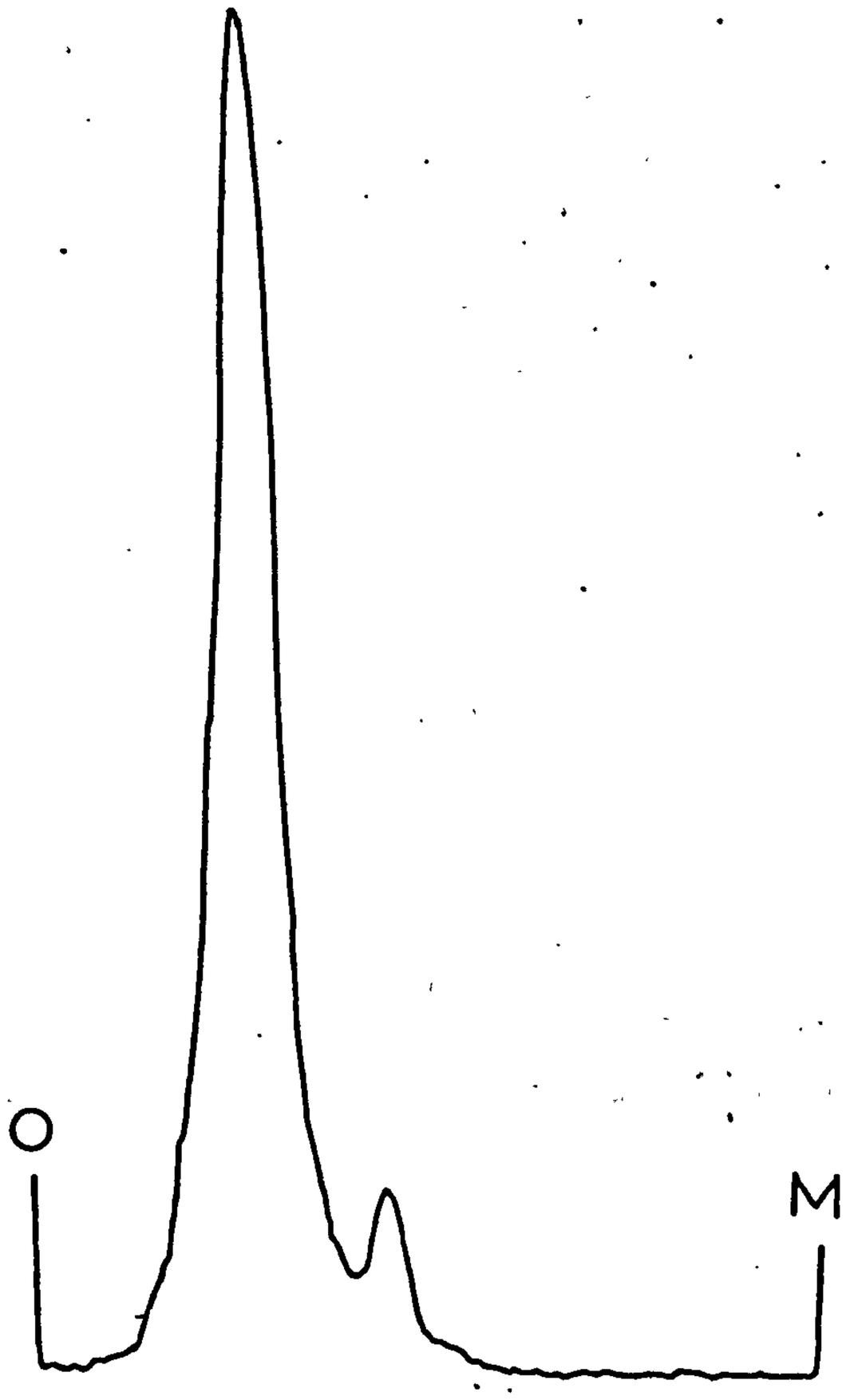


Figure 8.1. Scanning trace of 265 nm of 7.5% normal polyacrylamide gel after electrophoresis of purified erythrocyte hexokinase

O: Origin; M: bromophenol blue band after electrophoresis (+), (-): anode, cathode.

and 1 mM EDTA, prior to electrophoresis, did not alter the protein or activity pattern obtained on electrophoresis on 7.5% polyacrylamide.

(b) Molecular size

(1) Molecular weight and diffusion coefficient ($D_{20.w}$) by analytical gel filtration

A sample of 7 units of purified erythrocyte hexokinase in 0.5 ml of buffer was applied to a Sephadex G-200 column calibrated with the protein markers shown in Table 9.1. Fractions of 1.4 ml were collected. The relation between the logarithm of the molecular weight of markers and their elution volume is shown in Figure 9.3. The relation between the reciprocal of the diffusion coefficients of the markers and their elution volume is shown in Figure 9.4. The molecular weight was estimated as 108,500 and the $D_{20.w}$ as 50 $\mu m^2/s$. The diffusion coefficient corresponds to a Stoke's radius of 42.5 A^o.

The molecular weight of purified human erythrocyte hexokinase was found to be higher (132,000) by gel filtration on Sephadex G-200 and Ultrogel AcA 44 by Rijksen and Staal (1976a). The molecular weight of the same enzyme in haemolysates was found, by gel filtration on Sephadex G-200, to be in the same range i.e. 103,900 and 107,700

for HK I and HK II respectively by Rogers et al. (1975b).

(2) $S_{20.w}$ value by preparative ultracentrifugation

The sample applied to sucrose density gradient preparative ultracentrifugation contained 5 units of purified erythrocyte hexokinase, 2.5 mg of horse heart cytochrome C and 120 units of rabbit muscle lactate dehydrogenase. The concentration of cytochrome c was measured by absorbance at 550 nm. The activity of lactate dehydrogenase was assayed by the change of absorbance at 340 nm after adding 20 µl of each fraction in 3 ml of 0.1M sodium phosphate buffer pH = 7.4 containing 0.58 mM NADH and 1.8 mM sodium pyruvate. The sucrose concentration of the fractions was measured by a Bellingham & Stanley Ltd (London) refractometer.

By plotting $s_{20.w}$ values versus the sucrose concentration at the peak of each protein (Figure 8.2) an $s_{20.w}$ value of 6.1 was found for erythrocyte hexokinase assuming $s_{20.w}$ values of 2.1 S for cytochrome c (Atlas and Farber, 1956) and 7.6 S for lactate dehydrogenase (Jaenicke and Knof, 1968).

(3) Sedimentation velocity patterns. Effect of glucose 6-phosphate

The sedimentation velocity profile of purified erythro-

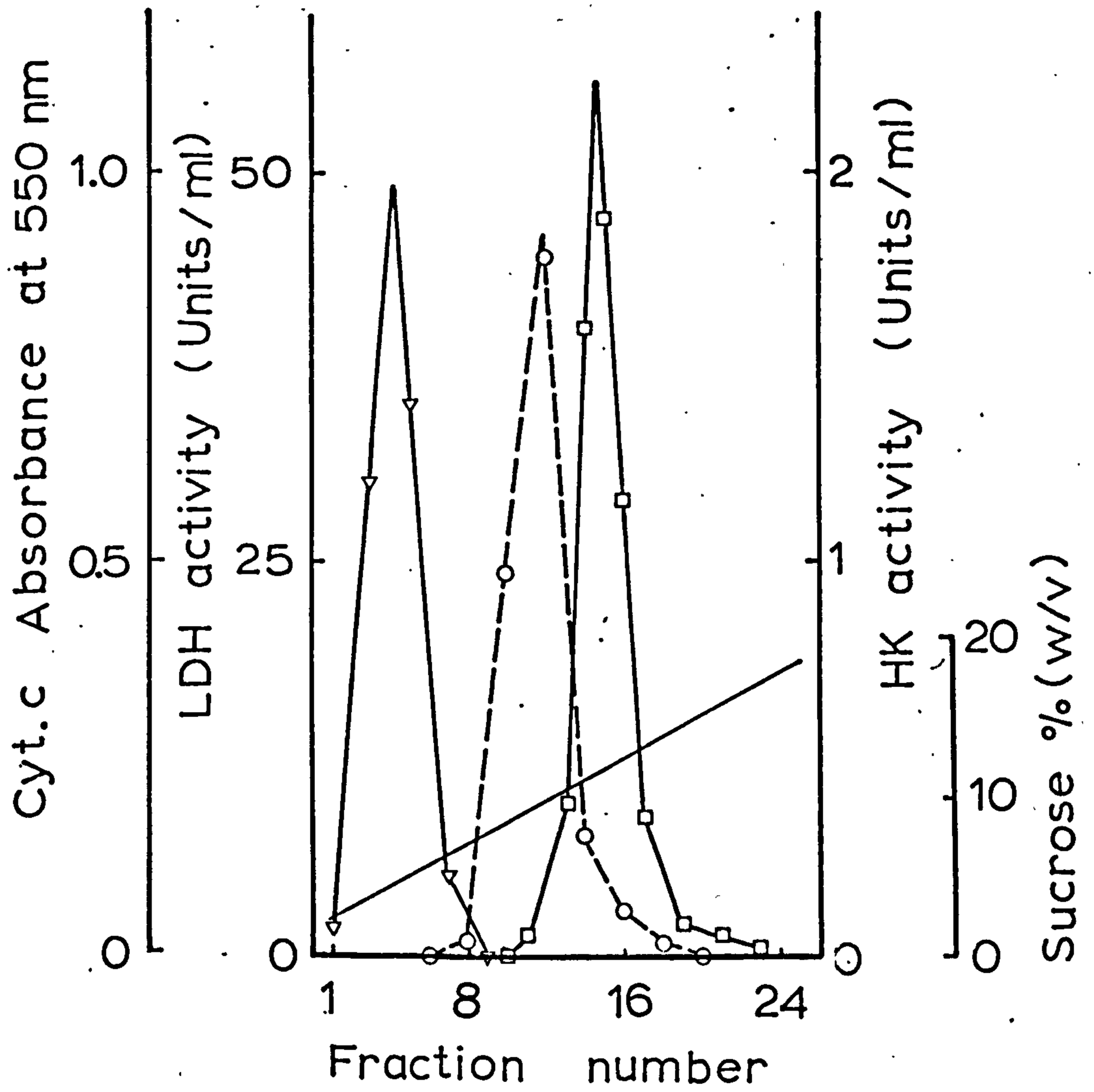
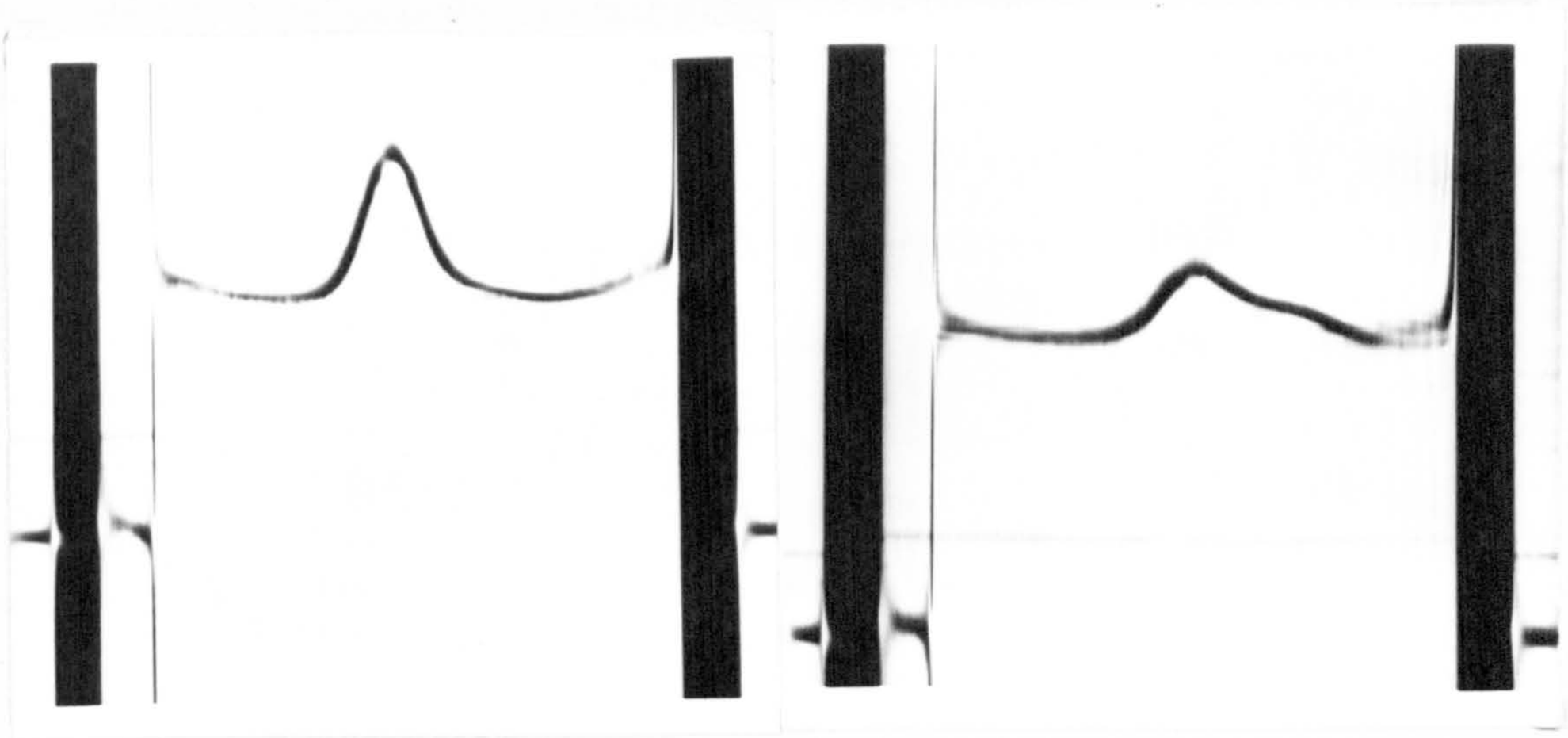


Figure 8.2. Sucrose gradient preparative ultracentrifugation of erythrocyte hexokinase

(▽——▽) Absorbance at 550 nm of cytochrome c; (□——□) Activity of lactate dehydrogenase; (○--○) Activity of hexokinase.



(A) (B)

Figure 8.3. The effect of glucose 6-phosphate on the sedimentation pattern of purified erythrocyte hexokinase

(A) The buffer was phosphate pH = 7.0, I = 0.1 containing 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercapto-ethanol.

(B) The buffer was Tris-HCl pH = 8.0, I = 0.01 containing 0.1 M KCl, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 1 mM glucose 6-phosphate.

The protein concentration was 4 mg/ml. Photographs were taken 90 minutes after reaching the speed of 59,780 rpm at a schlieren plate angle of (A)45°(B)40°. The temperature was 10°C.

cyte hexokinase in Figure 8.3 (A) showed a single symmetrical peak with a faster moving trace. The $s_{20.w}$ value of the major peak was 5.45 S. Although this value can not be referred to hexokinase since the sample was not pure, it is interesting that the value found is very near to that of heart hexokinase (5.5 S) under the same buffer conditions. However the value of 6.1 S found by preparative ultracentrifugation is much higher.

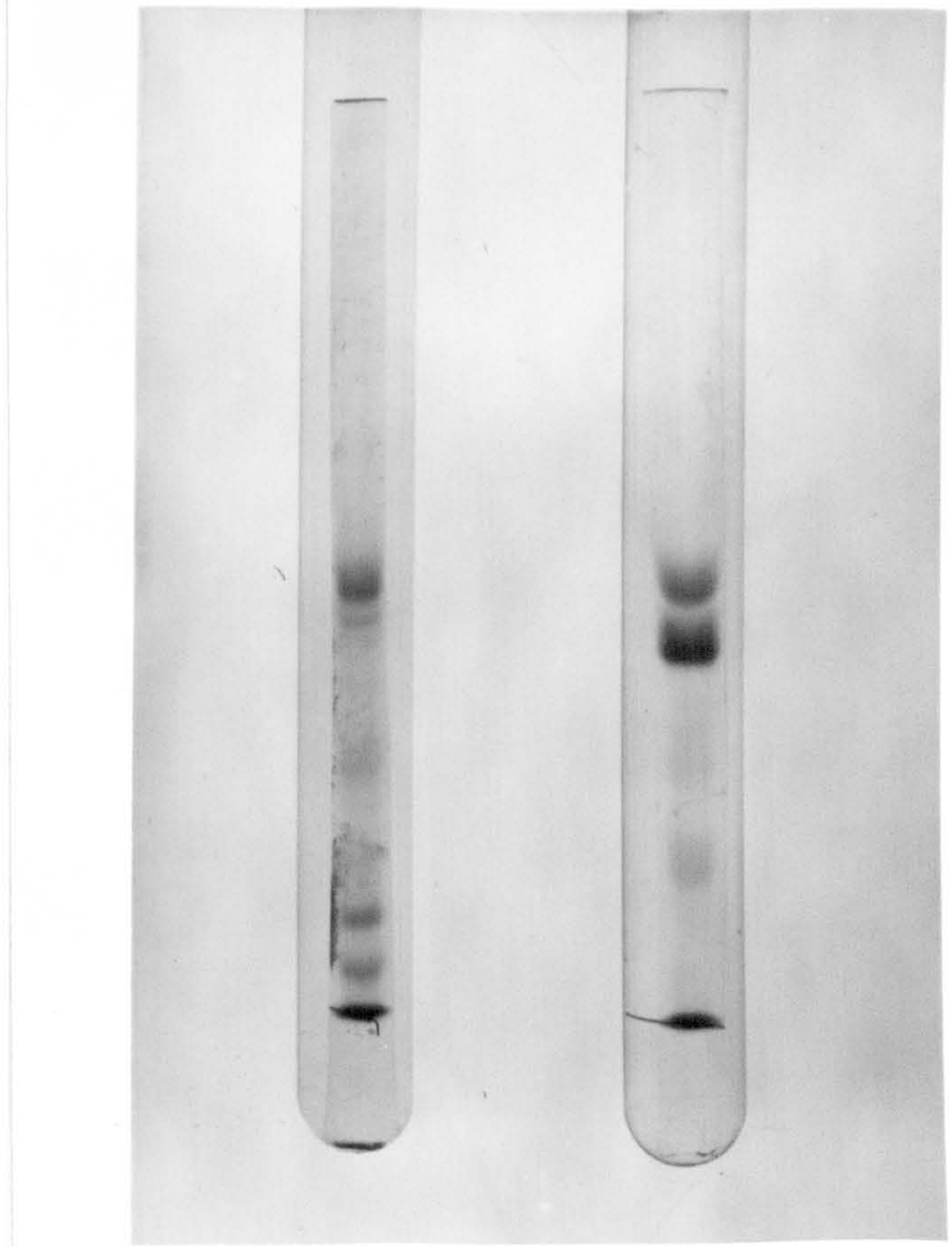
If the above sample of erythrocyte hexokinase was dialysed against the buffer containing 1 mM glucose 6-phosphate, for 24 hours, molecular species of higher size were observed (Figure 8.3 B), evidently produced by aggregation. Thus the erythrocyte enzyme appears to aggregate in the presence of glucose 6-phosphate as found for the enzyme from heart (Chapter 9).

(4) <u>SDS-polyacrylamide gel electrophoresis</u>

50 µg of purified erythrocyte hexokinase were subjected to electrophoresis on 5% SDS-polyacrylamide gel (Figure 8.4,A).

The appearance of multiple bands with protein stain shows the heterogeneity of the sample, in accordance with the low specific activity of the purified material.

The slow main band has an identical relative mobility as the slower band observed for the purified heart hexokinase on electrophoresis under the same conditions (Fig-



(A) (B)

Figure 8.4. 5% SDS-polyacrylamide gel electrophoresis of erythrocyte and heart hexokinase

(A) 50 µg per tube purified erythrocyte hexokinase

(B) 40 µg per tube purified heart hexokinase

A piece of wire marks the position of bromophenol blue band after the electrophoresis. Staining was for proteins.

ure 8.4,B). The results suggest that the slow main band in the erythrocyte sample represents hexokinase.

CHAPTER 9

Heart hexokinase

(a) Electrophoresis

Electrophoretic investigations on mammalian heart tissues have shown the presence of hexokinase type I only, for man (Neumann et al., 1974; Ritter et al., 1974; Kamel and Schwarzfischer, 1975; Rogers et al., 1975b), for yellow baboon (Papio cynocephalus), for green monkey (Cercopithecus aethiops) (Ritter et al., 1974) and for pig (Easterby and O'Brien, 1973). Besides HK I, traces of HK II and HK III were observed for guinea pig heart (Faulkner and Jones, 1976). However for rat heart, by DEAE-cellulose chromatography, HK I was found to represent only 35% of the total soluble hexokinase, the rest characterized as HK II (Katzen et al., 1970).

Electrophoretic results shown below suggest the presence of one isoenzyme, presumably type I, in human heart as well as the electrophoretic homogeneity of the purified enzyme.

(1) Starch gel electrophoresis

Small paper-strips were moistened with a purified heart hexokinase solution containing 9 mg of protein per

ml of solution. A single anodal band was stained with protein stain under the electrophoretic conditions shown in Table 9.1.

In every electrophoretic experiment there was a coincidence of activity and protein stain. However, the activity stain was too diffuse to be a reliable criterion of purity. The fact that a diffuse band of activity was observed only on starch gels but not on cellulose acetate strips or polyacrylamide gels may indicate a possible interaction of purified hexokinase with starch gel.

One anodal band of hexokinase, 1.3 cm from the origin, was also observed by Neumann et al., (1974) with 20 mM barbital-HCl buffer, pH = 8.6, after a 5 hours electrophoresis at 250 V. Rogers et al., (1975b), found in homogenates of human heart, two bands of HK I with the Tris-EDTA-borate-Mg buffer system, pH = 8.6. However, with the same electrophoretic conditions two bands were also observed with a heart extract in this project, but only one band was obtained with the purified material.

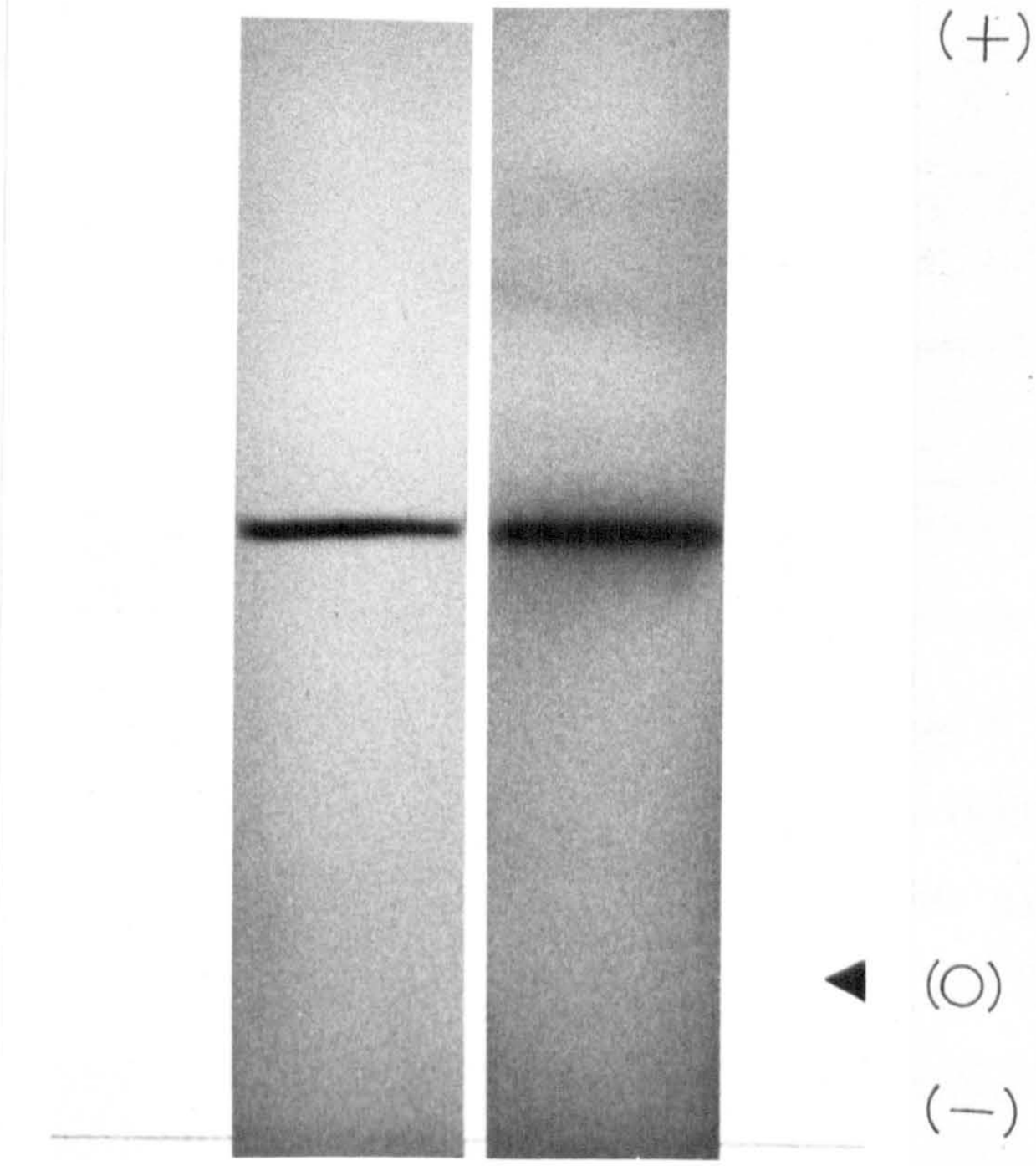
(2) Cellulose acetate electrophoresis

With heavy protein loading (45 µg of purified heart hexokinase per cellulose acetate strip) a single protein band was observed coinciding with the enzyme activity band (Figure 9.1). This band moved about 5 cm towards the

Table 9.1. Starch gel electrophoresis of heart hexokinase

Buffer system	Distance from origin (cm) Hexokinase	CO-Hemoglobin	Time (hours)	Electric field
Tris-EDTA-Borate pH = 8.6	+2.8	+5.4	5	480 V
Tris-EDTA-Borate-Mg pH = 8.6	+1.3	+5.5	20	85 V
Potassium phosphate pH = 7.0	+0.35	-0.25	5	50 mA

+, -; indicate movement towards the anode or cathode.



(A) (B)

Figure 9.1. Electrophoresis of 45 µg of heart hexokinase on cellulose acetate strips at pH = 8.6

A : Strip stained for protein.

B : Strip stained for hexokinase activity.

O, (+), (-) : Origin, anode, cathode.

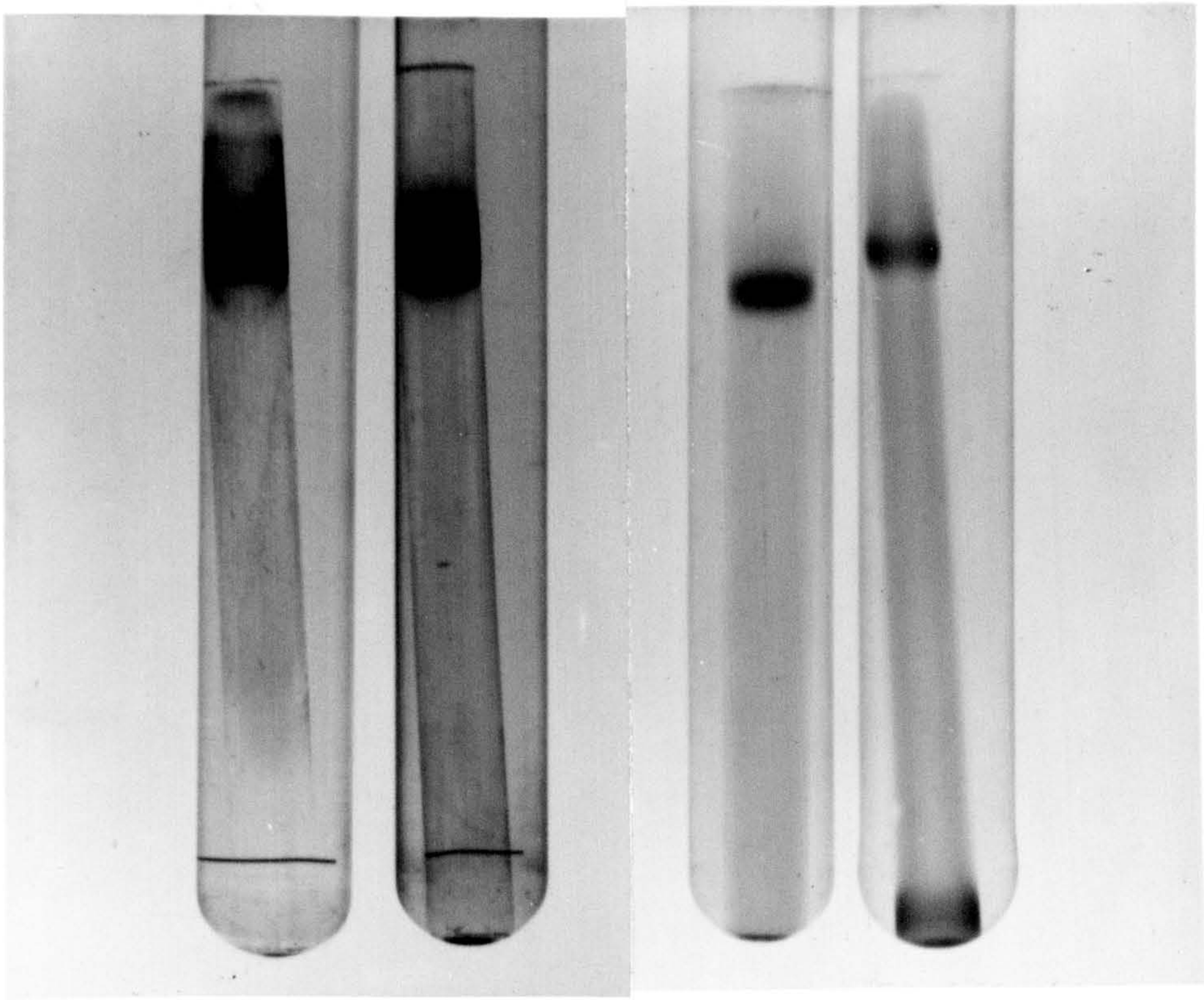
anode under the present experimental conditions (Chapter 7). Controls omitting ATP from the staining solution, were negative.

(3) Polyacrylamide gel electrophoresis

The electrophoretic profile of heart hexokinase with 7.5% normal polyacrylamide gel electrophoresis is shown in Figure 9.2.

Either with low protein loading (5 µg/tube) for better resolution or with heavy protein loading (40 µg/tube) for detecting minor protein components and enzyme bands with low hexokinase activity, a single band stained for hexokinase activity was observed corresponding to the single band stained for protein. The same profile was also observed with 5% polyacrylamide gels, except that the hexokinase band travelled double the distance from the origin and was broader.

No difference was observed in the electrophoretic profile with either of the staining methods for hexokinase activity i.e. incorporation of G6PD into the gel or into the staining solution. However the former method appeared more sensitive and gave staining in depth whereas the latter stained a faint ring outside the gel because G6PD could not diffuse into the gel. Controls omitting ATP from the staining solution, were negative.



(A) (B) (C) (D)

Figure 9.2. Electrophoresis of heart hexokinase on 7.5% normal polyacrylamide gel

A and B : 40 µg of protein loaded per tube.

C and D : 5 µg of protein loaded per tube.

A and D : stained for hexokinase activity with G6PD incorporated into the gel.

B and C : stained for protein.

Pieces of wire mark the position of bromophenol blue band after the electrophoresis.

(4) Electrophoresis as a criterion of purity

Electrophoresis with heavy protein loading may reveal the presence of small quantities of impurities differing mainly in charge from the protein of interest.

Starch gel electrophoresis with paper-strips impregnated with a 9 mg/ml solution of purified heart hexokinase, or cellulose acetate electrophoresis with 45 µg of protein per strip, or polyacrylamide gel electrophoresis with 40 µg per gel showed consistently a single protein band. This indicates the electrophoretic homogeneity of the sample.

(b) Molecular Size

(1) Initial estimation of molecular weight and diffusion coefficient, $D_{20.w}$, by analytical gel filtration

A sample of 0.5 ml, was applied on a Sephadex G-200 column, containing the following markers: 15 mg/ml γ-globulins, 15 units/ml lactate dehydrogenase, 40 mg/ml serum albumin, 20 mg/ml ovalbumin and 3 mg/ml blue dextran. Molecular weights and diffusion coefficients of the protein markers, derived from gel filtration experiments are given in Table 9.2. Blue dextran was used for the estimation of the void volume. The concentration of ovalbumin, serum albumin and γ-globulin in the fractions was measured by the absorbance at 280 nm. The concentration of blue-

Table 9.2. Protein markers for gel filtration

Protein	Source	Molecular weight x 10^3	Diffusion Coefficient ($D_{20.w}$) $\mu m^2/s$	Reference
γ-Globulins	Bovine	205	41	Andrews, (1965)
Lactate dehydrogenase	Pig heart	147	-	Determann and Mättner, (1969)
Albumin	Bovine serum	67	59	Andrews, (1965)
Ovalbumin	Egg	41	78	Andrews, (1965).

dextran was measured by the absorbance at 620 nm and the activity of lactate dehydrogenase by the assay method used after sucrose gradient centrifugation (Chapter 8).

A sample of 0.5 ml containing 20 units/ml of purified heart hexokinase was chromatographed and fractions of 1.6 ml were collected.

The relation between the logarithm of the molecular weight of markers and their elution volume is shown in Figure 9.3. The relation between the reciprocal of the diffusion coefficients of the markers and their elution volume is shown in Figure 9.4. By linear regression the molecular weight was found equal to 106,000 and the diffusion coefficient ($D_{20,w}$) equal to 51 $\mu m^2/s$. The latter value corresponds to a Stoke's radius of 42 A^o.

Neumann et al. (1974) reported an apparent molecular weight for purified heart hexokinase by gel-filtration on Sephadex G-200 of 111,000 which is in approximate agreement with the present results.

(2) Gel filtration as a criterion of purity

A sample of 1.4 ml of purified heart hexokinase with a protein concentration of 18 mg/ml was applied on a Sephadex G-200 column. The elution profile is shown in Figure 9.5. Fractions of 4 ml were collected.

Within the sensitivity of the measurement of absorb-

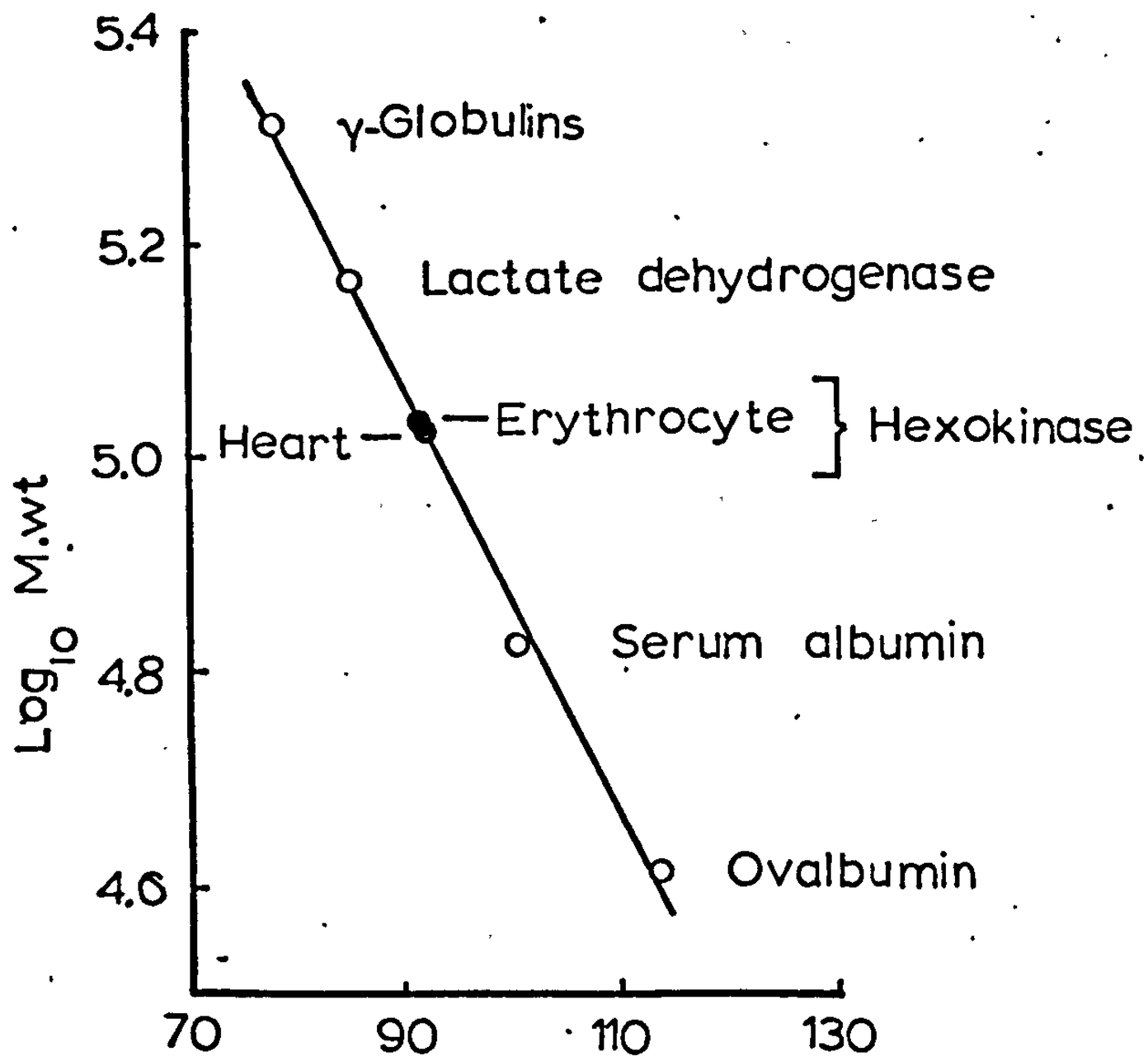


Figure 9.3. Empirical method for estimating the molecular weight of hexokinase from heart and erythrocyte

Plot of Log_{10} molecular weight against elution volume for standard proteins on a Sephadex G-200 column.

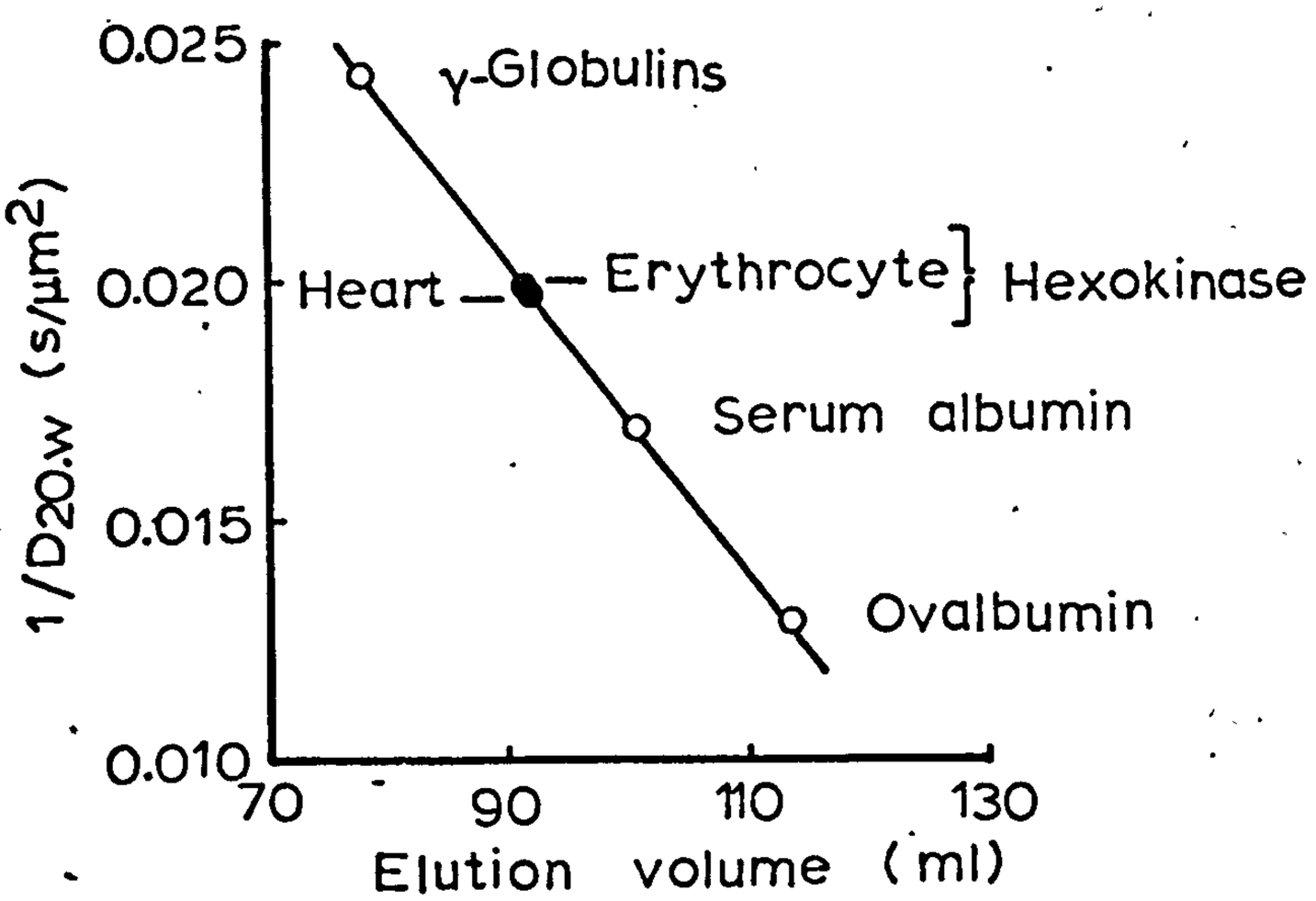


Figure 9.4. Diffusion coefficient for erythrocyte and heart hexokinase from gel-filtration

Plot of $1/D_{20.w}$ versus elution volume for standard proteins after gel filtration on Sephadex G-200.

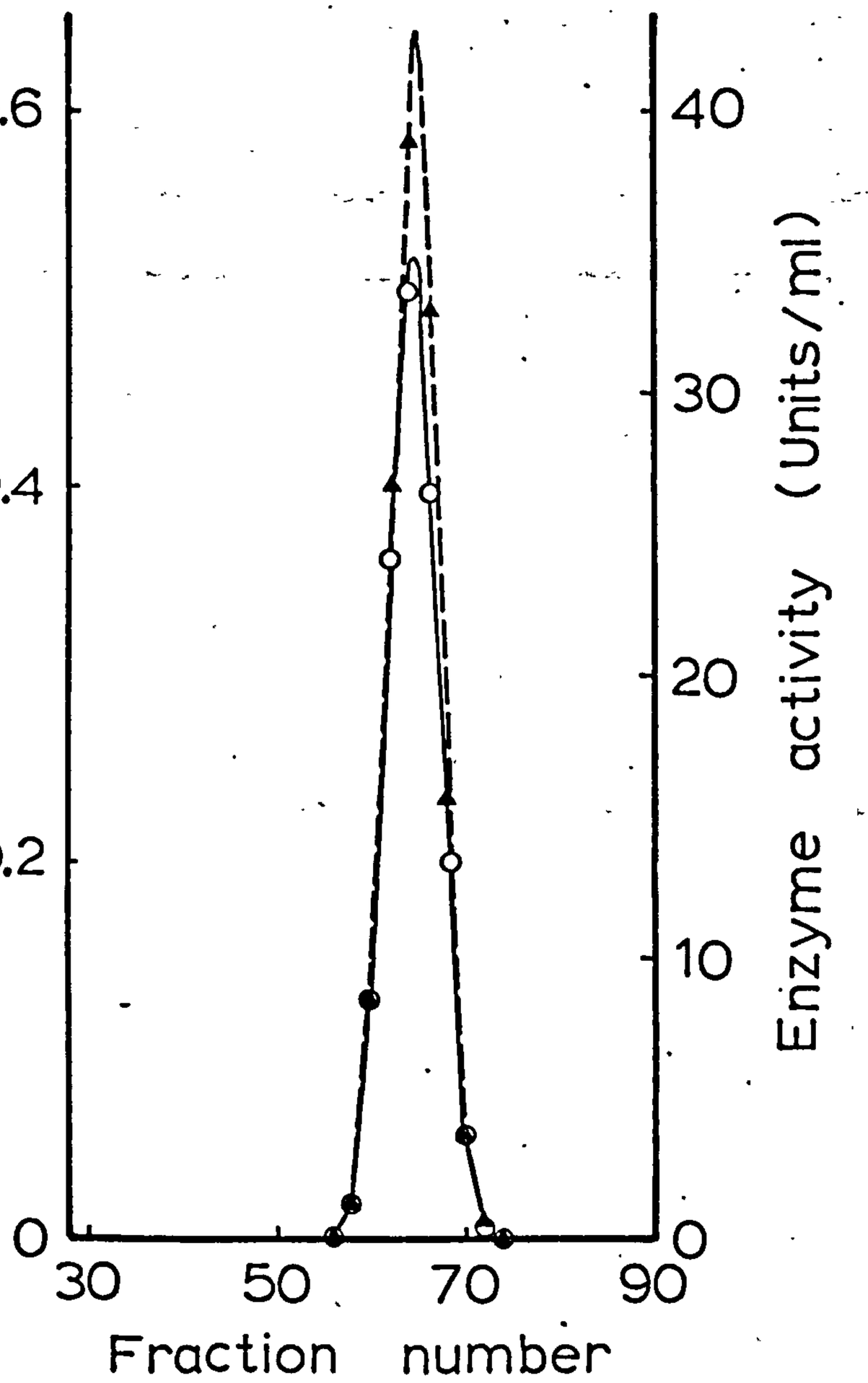


Figure 9.5. Elution profile from gel-filtration on Sephadex G-200 of purified heart hexokinase

(O——O) Absorbance at 280 nm; (▲---▲) Enzyme activity. Fractions of 4 ml were collected.

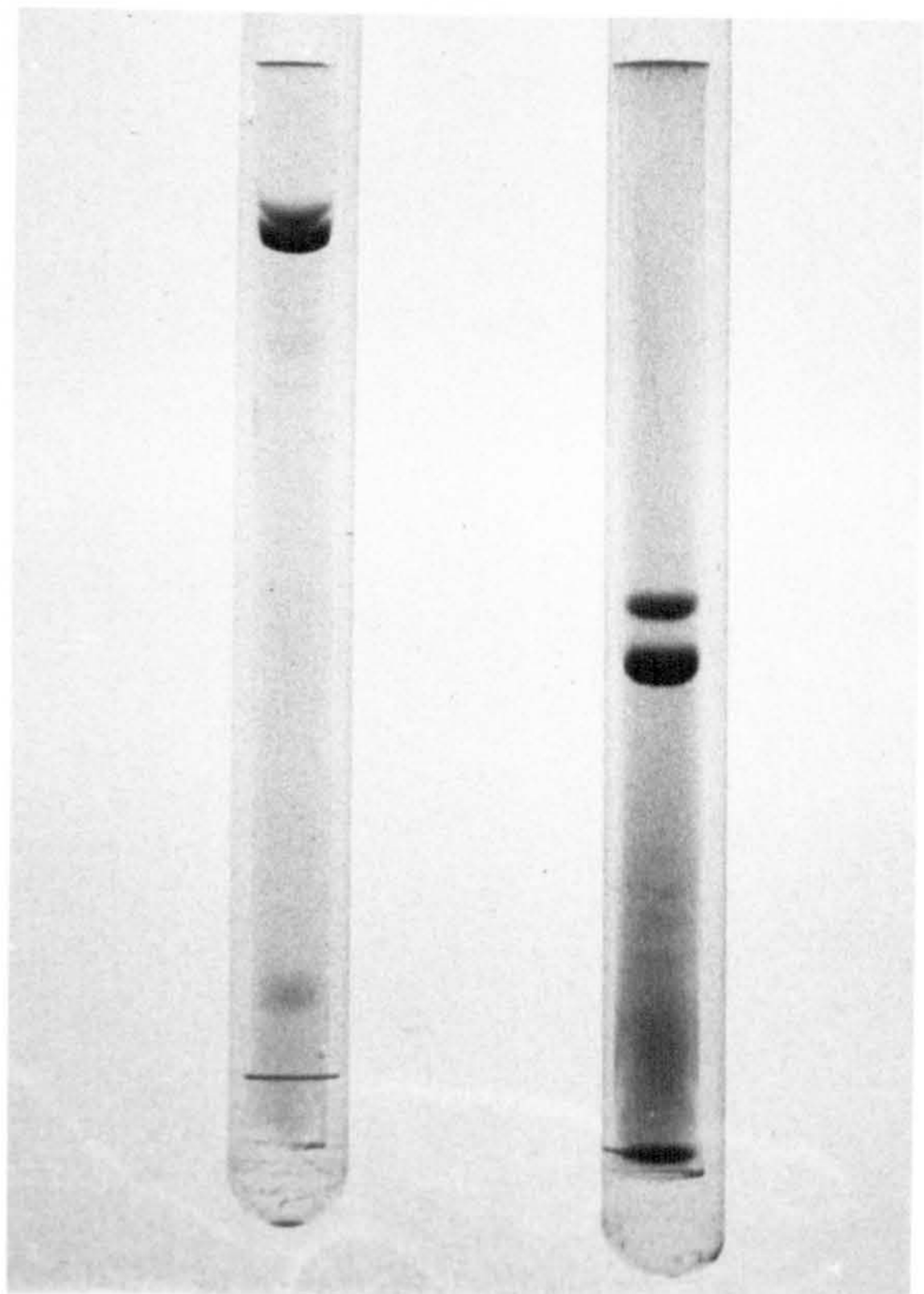
ance at 280 nm a single protein peak was found coinciding with the enzyme activity. The specific activity was constant in the fractions tested, except at both edges of the peak where protein measurements were low and thus unreliable. Within the limits of resolution of this method the sample appears homogeneous with respect to size.

(3) Estimation of molecular weight by SDS-polyacrylamide electrophoresis

Figure 9.6. shows 5 and 10% SDS-polyacrylamide gel electropherograms of purified heart hexokinase with protein loading of 6 μg/gel. For the estimation of the molecular weight of hexokinase the 5% polyacrylamide gel was preferred for its better resolution and thus more accurate estimation of molecular weight.

The protein markers myosin, β-galactosidase, phosphorylase a, bovine serum albumin, pyruvate kinase and ovalbumin were run separately. The molecular weights assumed for the markers are shown in Table 9.3. Graphs of the logarithm of molecular weights of the markers against their relative mobility for 5 and 10% SDS-polyacrylamide gels are shown in Figures 9.7. and 9.8. respectively.

With linear regression the two bands were found to correspond to molecular weights of 124,000 and 109,000 at 5% polyacrylamide concentration. The two bands thus



(A) (B)

Figure 9.6. SDS-polyacrylamide electrophoresis of 6 µg heart hexokinase

(A) 10% SDS-PAGE

(B) 5% SDS-PAGE

Table 9.3. Protein markers for SDS-polyacrylamide electrophoresis

Protein	Source	Molecular weight x 10^3	Reference
Myosin	Rabbit muscle	220	Woods et al., (1963), Slayter and Lowey, (1967), Ullmann et al., (1968).
β-Galactosidase	E.coli	130	Seery et al., (1967), Ullmann et al., (1968)
Phosphorylase α	Rabbit muscle	100	Cohen et al., (1971)
Albumin	Bovine serum	68	Tanford et al., (1967)
Pyruvate kinase	Rabbit muscle	57	Steinmetz and Deal, (1966)
Ovalbumin	Egg	43	Castellino and Barker, (1968).

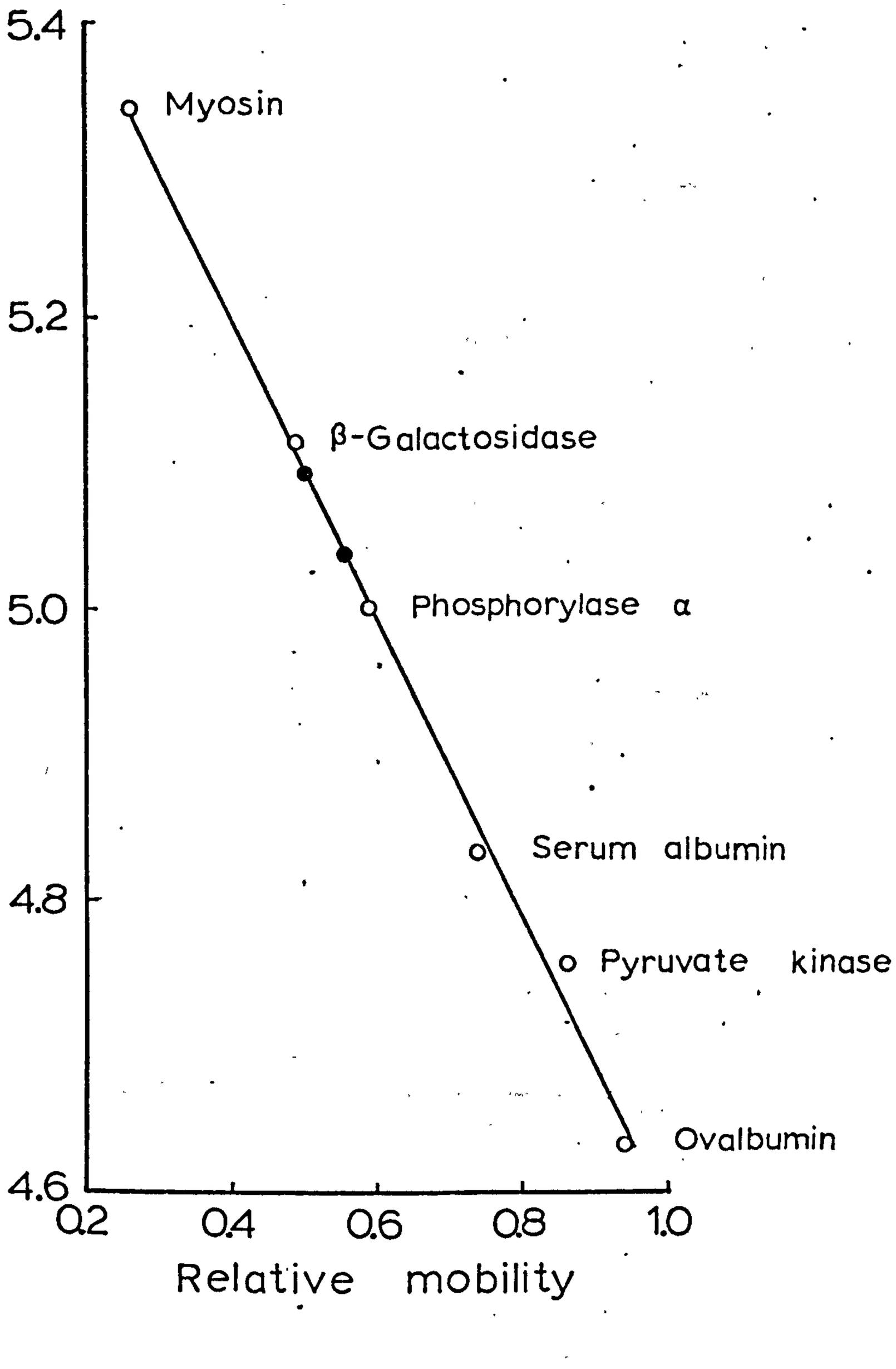


Figure 9.7. Graph of the logarithm of molecular weight against relative mobility for the standard proteins on 5% SDS-polyacrylamide gel electrophoresis

Full circles represent the two bands of the heart hexokinase sample.

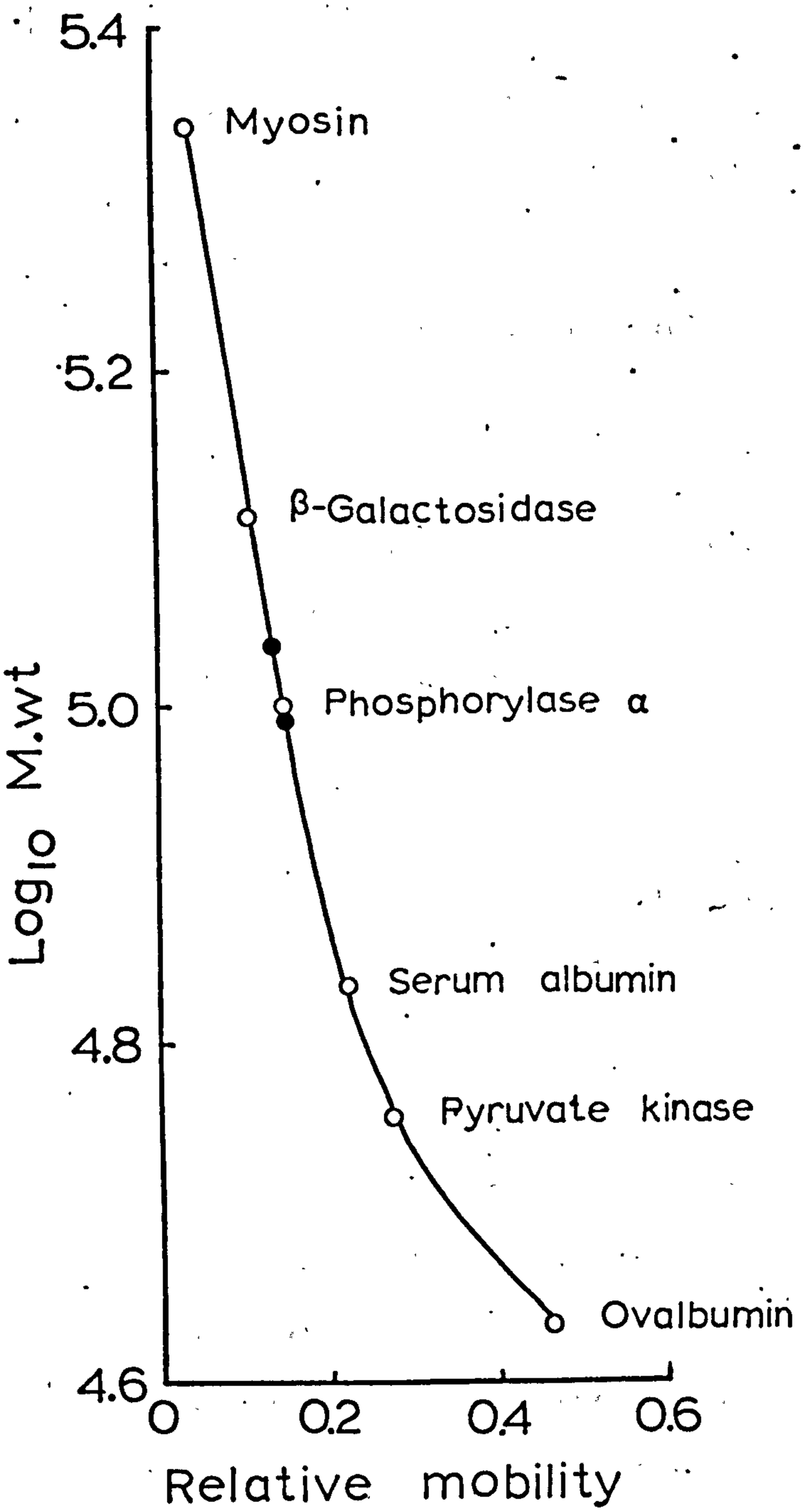


Figure 9.8. Graph of log molecular weight against relative mobility for the standard proteins on 10% SDS-polyacrylamide gel electrophoresis

Full circles represent the two bands of the heart hexokinase sample.

differed by 15,000. With 10% polyacrylamide concentration however, both bands corresponded to smaller molecular weights (Table 9.4).

The observation of two bands instead of the expected one, is difficutl to interpret. It is generally accepted that low Km mammalian hexokinases (HK I and HK II) are monomeric with a molecular weight of approximately 100,000 as measured under denaturing conditions (Easterby, 1971; Chou and Wilson, 1972; Easterby and O'Brien, 1973; Holroyde and Trayer, 1976).

However, some divergencies to this pattern have been reported. Easterby (1971) observed with porcine heart hexokinase two bands corresponding to molecular weights of 92,000 and 100,000 after SDS-polyacrylamide electrophoresis. These bands were reduced to one band of 97,000 molecular weight if the enzyme was heat-treated (to 100°C for 5 min) prior to dialysis against SDS-containing buffer. Craven and Basford (1974) reported that rat brain hexokinase solubilised by glucose 6-phosphate and purified by DEAE-cellulose chromatography showed, on SDS-polyacrylamide gel electrophoresis, a major band of 130,000 and a minor band of 100,000 molecular weight when the enzyme was treated at 45°C with 1% (w/v) SDS and 0.2% (v/v) 2-mercaptoethanol. However if the same sample was pretreated with 2% (v/v) 2-mercaptoethanol for 30 minutes in boiling water before the addition of 1% (w/v) SDS, a peptide of 45,000

Table 9.4. Estimation of molecular weight of heart hexokinase by SDS-polyacrylamide electrophoresis

Large component ($M.wtx10^3$)	Small component ($M.wtx10^3$)	Difference ($M.wtx10^3$)	Acrylamide concentration %
124	109	15	5
124	110	14	5
125	108	17	5
117	98	19	10

appeared suggested as a subunit of hexokinase.

Monakhov et al., (1975) showed that four isoenzymes of soluble human gastric mucosa hexokinase purified by polyacrylamide gel electrophoresis had molecular weights ranging from 112,000 to 125,000 as found by density gradient preparative ultracentrifugation. Treatment of each isoenzyme with 1% SDS and boiling for 3 minutes led to the appearance of two intense bands on SDS-polyacrylamide gel electrophoresis corresponding to molecular weights of 60,000 and 96,000, whereas if 8 M urea was added on top of SDS only one band of 60,000 molecular weight appeared.

The two bands of purified human heart hexokinase shown in Figure 9,9 may represent either two hexokinase molecules differing in size, or hexokinase and a major impurity. If both bands represent hexokinase, the smaller one could be a degraded form of the other. Alternatively the larger species could include a non-protein moiety such as lipid or carbohydrate.

Moreover one can not exclude the creation of artifacts during the preparation of samples and electrophoresis. Partial denaturation of a protein molecule may lead to incomplete binding of SDS to it and thus to electrophoretic mobility similar to a higher protein species. Ammonium persulphate used for the initiation of polymerization of the gelling solution is reported to produce artifacts with

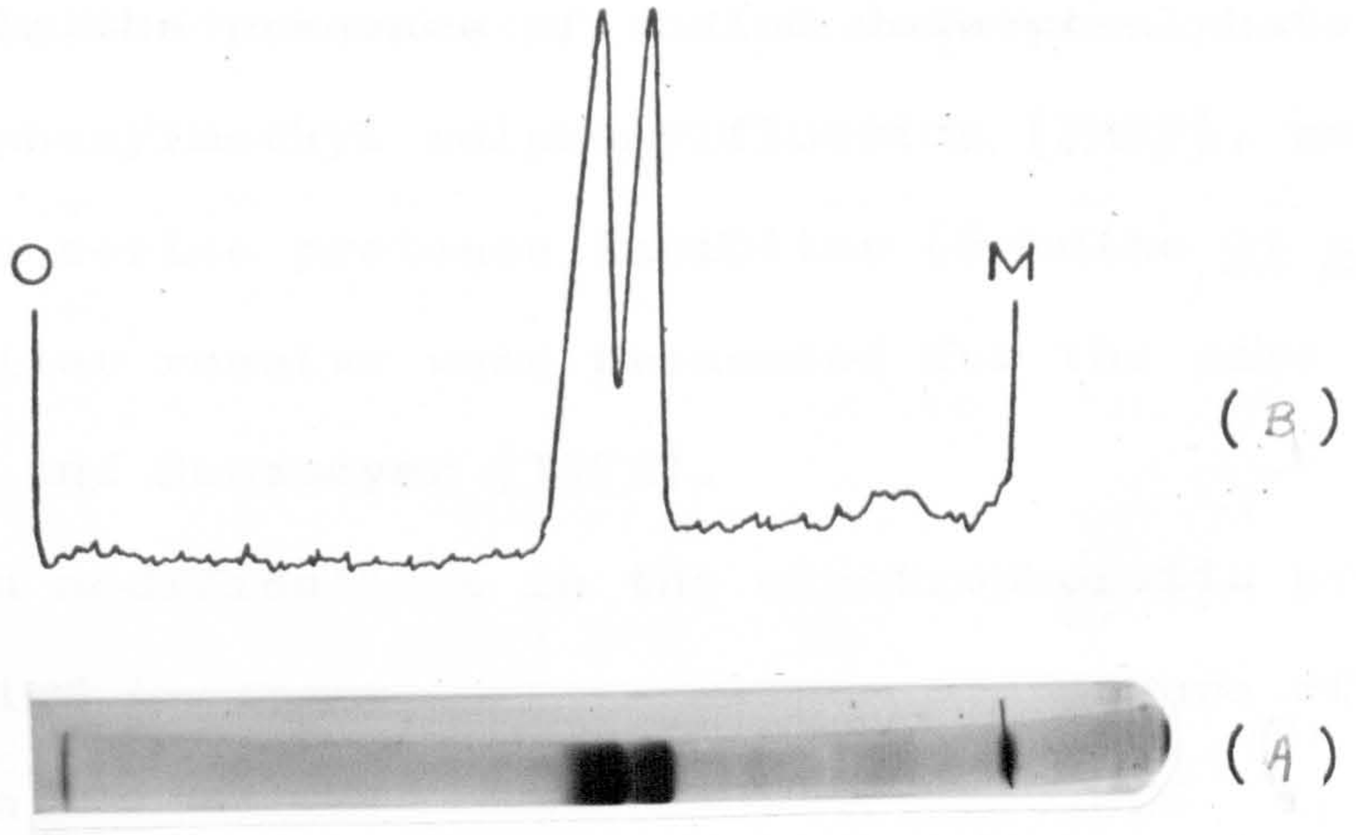


Figure 9.9. 5% SDS-polyacrylamide electrophoresis of 45 µg of heart hexokinase

(A) Staining for proteins.

(B) Scanning at 265 nm. Origin (O) and position of bromophenol blue band (M).

normal polyacrylamide electrophoresis (Mitchell, 1967; Shapiro et al., 1967; Brewer, 1967; Schyns, 1968).

Pringle (1970) reported that proteolysis of yeast hexokinase in the presence of sodium dodecylsulphate was avoided by phenylmethyl sulphonylfluoride (PMSF), an irreversible serine protease inhibitor (Schulze et al., 1967). Similar results were presented for the same enzyme by Easterby and Rosemeyer (1972).

Certain modifications in the electrophoretic procedure were tried in order to investigate the nature of the two bands on SDS-polyacrylamide electrophoresis

(i) Prerun of gels for 2 hours with a constant current of 2 mA/tube.

(ii) Photopolymerization of gelling solution with 5 µg/ml riboflavin.

(iii) Blockage of sulfhydryl groups of the sample with iodoacetamide before boiling with the sample buffer.

(iv) Addition of 20 mM PMSF solution in 95% ethanol to the sample, to a final concentration of 1-2 mM, prior to the boiling with the sample buffer.

(v) Addition of PMSF to the homogenate of heart tissue (Step 1 of the respective purification

procedure) to a final concentration of 1-2 mM.

(vi) Dialysis of the sample against the sample buffer for 24 hours after its boiling with the same buffer.

(vii) Addition of 8 M urea to the sample buffer.

None of these modifications gave a change in the two band pattern. Also stain for glycoproteins was negative while stain for lipoproteins stained only a narrow band at the bromophenol blue-band position.

It is difficult to imagine that one of the two bands is an impurity since it must have followed closely hexokinase in all purification steps and also has an affinity for glucosamine bound on Sepharose. Interestingly the larger component corresponded to the main band seen for erythrocyte hexokinase on SDS-polyacrylamide gel (Chapter 8). The possible heterogeneity of hexokinase owing to attached lipid or carbonhydrate was ruled out with appropriate stains.

No half size subunits were observed with the purified human heart hexokinase in contrast to reports by Craven and Basford (1974) for rat brain hexokinase and Monakhov et al., (1975) for human gastric mucosa hexokinase. The findings of these authors should be interpreted with caution since experiments were performed with impure hexokinase samples.

(4) <u>SDS-polyacrylamide electrophoresis as a criterion of purity</u>

Heavy loading of SDS-polyacrylamide gels (45 mg of protein per tube) revealed two major bands and a minor one at the position of the bromophenol blue band, marked by a piece of wire, as seen in Figure 9.9(A). In Figure 9.9(B) the spectrophotometric tracing of absorbance at 265 nm is shown.

The denatured sample of purified heart hexokinase contained as discussed above, two protein components. The spectrophotometric trace shows that the components are present in approximately equal amounts in this preparation. In other preparations the amount of large component ranged from 25% to 55% of the total protein.

(5) <u>Estimation of partial specific volume,</u>

The partial specific volume of heart hexokinase was calculated from density measurements performed with the Anton Paar K.G. (Austria) digital precision density meter DMA 02D. The principle of this apparatus is based on the mechanical escillator technique described by Kratky <u>et al</u>. (1973).

For the density difference of two samples the following equation holds:

$$d_1 - d_2 = K(T_1^2 - T_2^2)$$

where d_1, d_2 are the densities of two samples

K is the constant of the apparatus

and T_1, T_2 are the respective times for 10^4 full periods of the vibrator (displayed in the apparatus in units of 10 μs) for the two samples.

Measurements were made at 10°C with a barometric pressure of 767.65 mm of mercury column (at 24°C). The density of dry air was assumed to be 0.001260 g/ml and that for water 0.999700 g/ml at 10°C. The solution of hexokinase had a protein concentration of 8.96 mg/ml in phosphate buffer pH = 7.0, I = 0.1 containing 0.2 M KCl, 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. The protein concentration was estimated from refractometric measurements as mentioned below.

From measurements of T for dry air and water, K was found equal to $3.250388.10^{-14}$ g/ml x s^2. From measurements of T for the water-buffer and water-protein solutions, the densities of buffer and protein solutions were found to be 1.016202 and 1.018536 g/ml respectively. A summary of T measurements is presented in Table 9.5.

Table 9.5.

Material	T (10^{-5}sec)	S.D.	S.E.	n
Dry air	5532018.5	1.91	0.96	4
Water	7830759.25	1.71	0.85	4
Buffer	7863108.5	4.80	2.40	4
Hexokinase solution	7867672.83	9.45	3.86	6

The partial specific volume was calculated from the following equation:

$$\bar{v}(c) = \frac{1}{d_1} \cdot \left(1 - \frac{d_2 - d_1}{c}\right)$$

where $\bar{v}(c)$ is the partial specific volume measured at a single solute concentration

d_1 is the density of the solvent in g/ml

d_2 is the density of the solution in g/ml

and c is the concentration of protein in g/ml.

Thus the partial specific volume was found equal to 0.728 ml/g under the above conditions.

The partial specific volumes of rat brain and porcine heart hexokinases calculated from their amino acid composition were found to be higher i.e. 0.74 (Chou and Wilson, 1972) and 0.737 ml/g (Easterby, 1971) respectively.

(6) Estimation of the extinction coefficient $\epsilon_{280\ nm}^{1\%,\ 1cm}$

The refractive index for hexokinase was assumed to be 1.6, a value typical for non-haem proteins. Proteins with a refractive index of about 1.6 have been reported to cause an average displacement of 4.1 fringes/mg/ml in a refractometer (Babul and Stellwagen, 1969). Thus by measuring the number of fringes crossed in traversing a protein-solvent

boundary, the concentration of a protein solution may be determined.

A Spinco model E ultracentrifuge equipped with Rayleigh interference optics was used as a refractometer. A sample approximately 5 mg/ml of the enzyme was dialysed exhaustively against phosphate buffer pH = 7.0, I = 0.1 containing 0.2 M KCl, 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. A boundary was formed between the protein solution and the dialysis buffer as described in Chapter 7. The boundary was allowed to diffuse at 6,995 r.p.m. at 10°C and interference photographs were taken over a period of 90 minutes.

The fringe displacement was measured using a twin co-ordinate travelling microscope and by counting the fringes crossed from solvent to solution. The hexokinase concentration was calculated using the value of 4.1 fringes displaced for each mg of protein per ml of solution. The absorbance of the hexokinase solution at 280 nm was measured prior to the ultracentrifuge experiment, using a Perkin-Elmer 124 double-beam spectrophotometer and 1 cm light-path cuvettes.

The absorbance at 280 nm combined with the protein concentration obtained by the fringe displacement in the ultracentrifuge, gave an extinction coefficient, $\varepsilon_{280\ nm}^{1\%,\ 1\ cm} = 6.0$. This value was then used for subsequent

measurement of protein concentration of heart hexokinase using the absorption at 280 nm.

Extinction coefficients reported for mammalian hexokinases are summarized in Table 9.6. These values although determined by various methods of protein estimation, vary only from 5.1 for brain hexokinase to 6.0 for the porcine heart enzyme. The higher value of 7.62 reported by Neumann et al. (1974) may be caused by impurities in their preparation.

The low absorbance at 280 nm is compatible with the relatively low content of aromatic amino acids as shown by amino acid composition studies of rat brain (Chou and Wilson, 1972) and porcine heart hexokinase (Easterby and O'Brien, 1973).

(7) Estimation of sedimentation coefficient, $s_{20.w}$, under various buffer conditions

In Table 9.7 results of sedimentation velocity ultracentrifugations are presented under various conditions of pH, ionic strength and protein concentration.

At pH = 7.0 no significant alteration of sedimentation coefficient was observed by doubling the protein concentration from 4 to 9 mg/ml.

The increase of ionic strength at pH = 7.0 from 0.1 to 2.1 resulted in a decrease of the sedimentation coeff-

Table 9.6. Extinction coefficients, $\varepsilon_{280\ nm}^{1\%,\ 1\ cm}$, of mammalian hexokinases

Source	$\varepsilon_{280\ nm}^{1\%,\ 1\ cm}$	Method of protein measurement	Reference
Human heart	7.62	Biuret	Neumann et al., (1974)
Porcine heart	6.0	Refractometric	Easterby and O'Brien (1973)
Rat brain	5.53	Lowry	Schwartz and Basford (1967)
Rat brain	5.1	Turbidimetric	Chou and Wilson (1972)
Bovine brain	5.5	Lowry	Redkar and Kenkare (1972)
Human heart	6.0	Refractometric	Present results.

Table 9.7. Effect of pH and ionic strength on the sedimentation coefficient

pH	Buffer	Protein concentration (mg/ml)	Correction factor	$s_{20,w}$ (to nearest 0.05)S
7.0	Phosphate I=0.1	4	1.340	5.50
7.0	Phosphate I=0.1	8	1.340	5.50
7.0	Phosphate I=0.1	9	1.340	5.50
7.0	Phosphate I=0.1+0.2M KCl	10	1.361	5.15
7.0	Phosphate I=0.1+2 M KCl	5	1.735	4.80
6.0	Acetate I=0.1+0.2 M KCl	6.5	1.416	5.85
8.0	Tris-HCl I=0.01+0.2 M KCl	6.5	1.324	5.30

All solutions contained 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Experiments were performed at 10°C.

ient from 5.5 to 4.8. If a dissociation to subunits was occurring, a much greater decrease of $s_{20.w}$ should be expected. So it is more likely that at high salt concentration the protein is preferentially hydrated, causing an increase in the partial specific volume ($\bar{U}$) for the protein. Since no allowance is made for this change of $\bar{U}$, the sedimentation coefficient appears to be low. This phenomenon has been reported to occur to other proteins, for instance serum albumin and aldolase (Reisler and Eisenberg, 1969).

The lowering of pH from 7.0 to 6.0 resulted in an increase of sedimentation coefficient from 5.15 to 5.85 at total ionic strength of 0.3. Since the schlieren peak at pH = 6 was a single symmetrical one, this increase can be either the result of a conformational change of the hexokinase molecule or of a rapid equilibrium between monomer and polymer(s).

(8) <u>Sedimentation velocity pattern as a criterion of purity</u>

Sedimentation relocity experiments are a useful guide to the purity of an enzyme preparation, although small amounts of impurities would not be visible using this technique.

Sedimentation experiments on the enzyme in buffers

containing 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol at 10°C, performed as described in Chapter 7, showed single, symmetrical boudaries over a range of pH and ionic strength. Figure 9.10 shows a typical sedimentation pattern obtained in phosphate buffer at pH = 7.0, I = 0.1.

The purified heart hexokinase sample appears homogeneous within the resolution and sensitivity limits of this type of experiment.

(9) Estimation of diffusion coefficient, $D_{20.w}$

Measurements of the $D_{20.w}$ by observing the diffusion of a boundary in a low gravitational field in the ultracentrifuge, gave a value of 47 $\mu m^2/s$ using two different buffer conditions (Table 9.8).

The graphical analysis of the diffusion data for the experiment in phosphate buffer pH = 7.0, I = 0.1 is shown in Figure 9.11.

The $D_{20.w}$ found in ultracentrifuge is slightly lower than the value found by gel filtration (51 $\mu m^2/s$). With $D_{20.w}$ equal to 47 $\mu m^2/s$, the calculated Stoke's radius is 45 A^o.

(10) Estimation of molecular weight by the combined sedimentation coefficient and diffusion coefficient values.

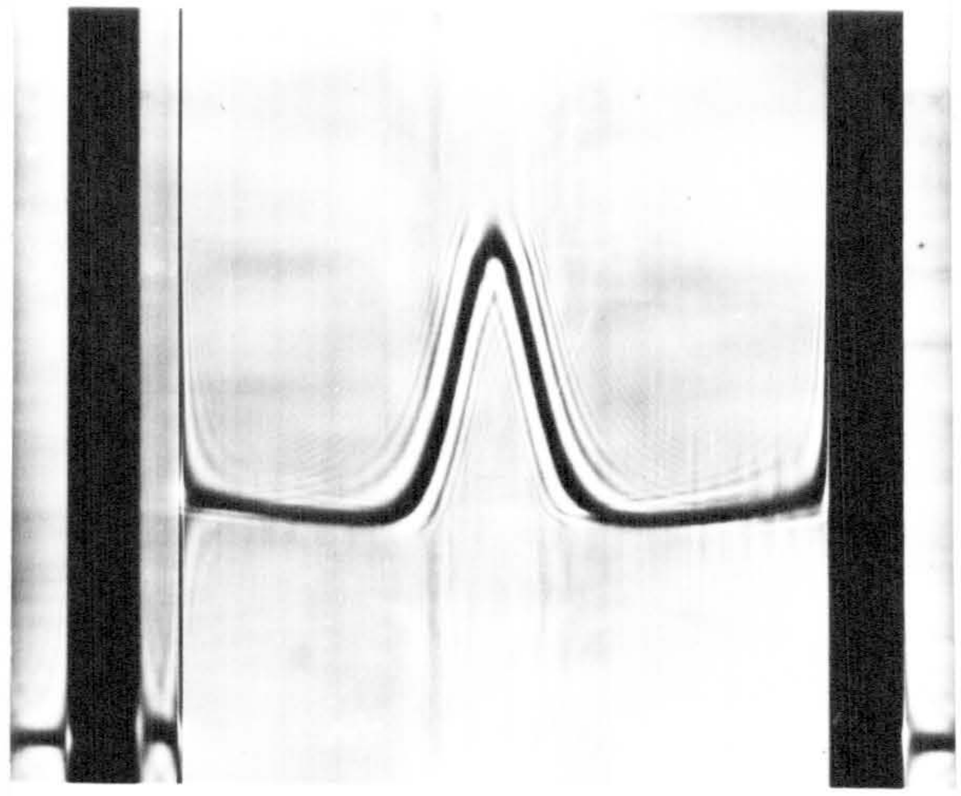


Figure 9.10. Sedimentation pattern of purified heart hexokinase

The protein was 8 mg/ml in potassium phosphate buffer pH = 7.0, I = 0.1 containing 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Migration is from left to right. The photograph was taken 90 min after reaching a speed of 59,780 rev/min. Temperature was 10°C. The schlieren plate angle was 55°.

Table 9.8. Estimation of diffusion coefficient, $D_{20.w}$, of heart hexokinase by boundary forming experiments

pH	Buffer	Protein concentration (mg/ml)	Correction factor	$D_{20.w}$ ($\mu m^2/s$)
7.0	Phosphate, I = 0.1	8	1.365	47
8.0	Tris-HCl, I=0.01+0.2M KCl	8	1.333	47

Buffers contained 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Experiments were performed at 10°C.

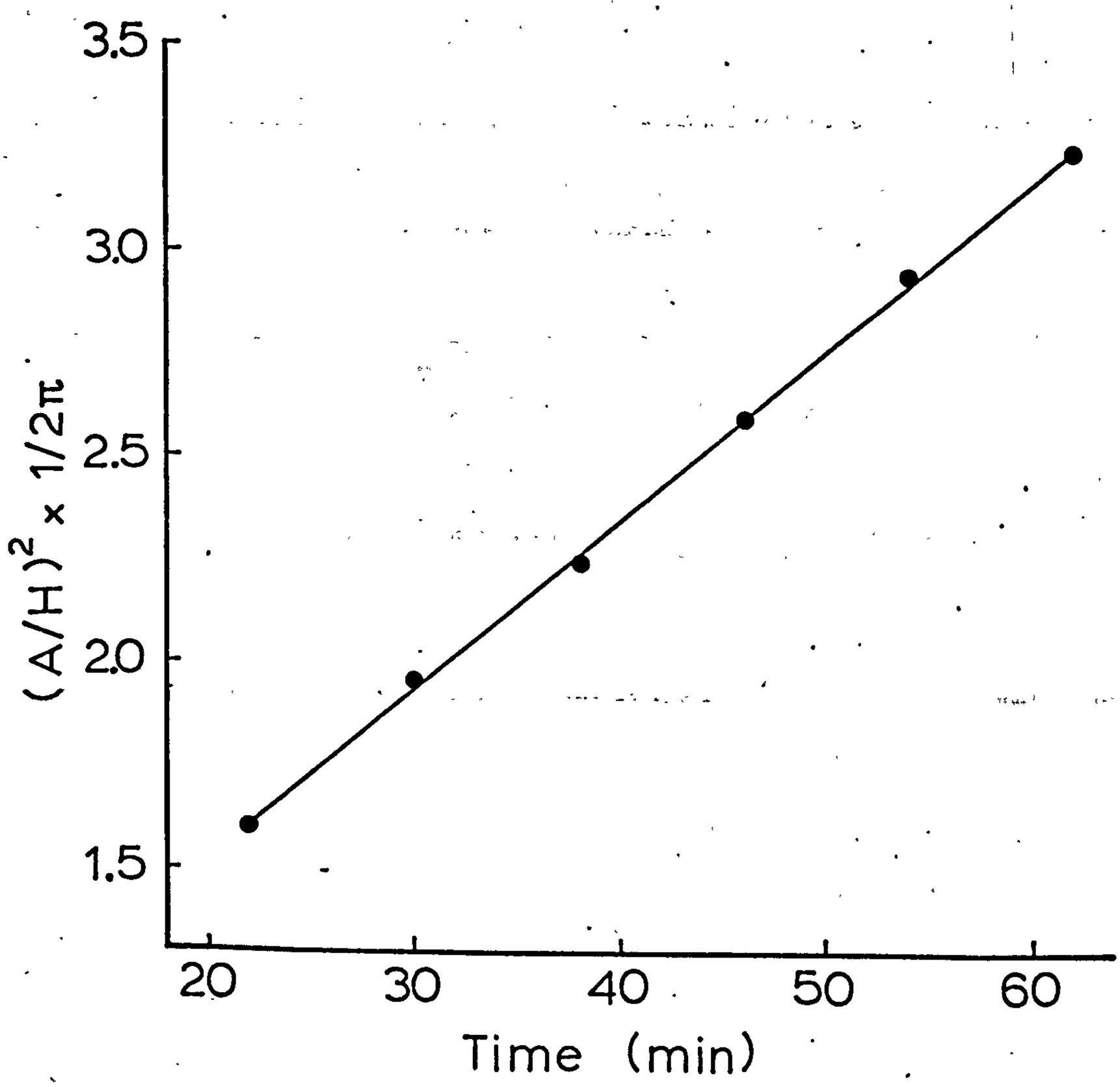


Figure 9.11. Analysis of the diffusion data for hexokinase in phosphate buffer pH = 7.0, I = 0.1

The square of the ratio of the area of the schlieren peak to its' height, over 2π, is plotted against time. Centrifugation was performed at 6,995 r.p.m. and at $10^{o}C$.

Assymetry of the hexokinase molecule

Measurement of the sedimentation coefficient and diffusion coefficient for hexokinase provided a molecular weight from the Svedburg equation:

$$M = \frac{RTs}{D(1-\bar{\upsilon}\rho)} \qquad \text{(Svedberg, 1925)}$$

Using values of 5.53 for $S_{20.w}$, 47 $\mu m^2/s$ for $D_{20.w}$ and 0.728 for $\bar{\upsilon}$, the molecular weight was estimated to be 105,000.

From the diffusion coefficient, an indication of the shape of the molecule is given by the frictional ratio f/fo (Svedberg and Pedersen, 1940). The observed molecular frictional coefficient is given by:

$$f = \frac{kT}{D}$$

The calculated molecular frictional coefficient for a sphere of molecular weight M is given by:

$$f_o = 6\pi\eta \left(\frac{3M\bar{\upsilon}}{4\pi N}\right)^{1/3}$$

In the present instance, ($M = 105{,}000$, $\bar{\upsilon} = 0.728$, $D_{20.w} = 47\ \mu m^2/s$), the frictional ratio f/fo is 1.46. For an hydration of 0.3 g water per g protein, the above ratio

indicates an axial ratio of 6:1 for a prolate ellipsoid according to Oncley (1941). Although the above treatment is an oversimplification, it suggests a certain degree of assymmetry in the hexokinase molecule. This assymmetry in molecular structure has also been found for G6PD (Cohen, 1969), 6-PGD (Pearse, 1972) and glutathione reductase (Worthington, 1974).

(11) Sedimentation equilibrium

Photographic results of two sedimentation experiments are shown in Figures 9.12 and 9.15. In all sedimentation equilibrium experiments 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol were present in solution. Also the centrifugation continued for 70 hours at a rotor speed of 6,995 r.p.m. and at a temperature of 10°C. The initial concentration was calculated from the corresponding boundary forming experiment.

Table 9.9 shows the results from four sedimentation equilibrium experiments. A general feature is the inconsistency of Mz and Mw values, indicating heterogeneity of material in these experiments.

A complication was observed with sedimentation equilibrium experiments No 2, 3 and 4 i.e. at the bottom of the cell a dense band was observed as can be seen from the photograph of Figure 9.15. Anomaly with the ultracentri-

fuge optics is unlikely as no such band was observed with the boundary forming experiments. This band may represent either very high polymers in solution or gelation.

The higher molecular species observed through the solution must represent polymers of hexokinase produced only during the sedimentation equilibrium experiment, since subsequent sedimentation velocity experiments did not reveal such protein species. Factors that would favour the production of polymers to a greater extent in sedimentation equilibrium than in sedimentation velocity experiments (once a tendency for polymerization exists) are the much longer time of the experiment, the lower pressure (due to lower speed) and the higher concentration of proteins towards the bottom of the cell.

The fact that polymers appear even at high salt concentration (2 M KCl) and in the presence of 2-mercaptoethanol, suggests that ionic or disulfide bands are unlikely to participate in polymerization. Therefore hydrophobic forces are more likely candidates.

Another common characteristic is that the six points measured from the meniscus fall into a line, as can be seen from graphical representations of two of the experiments (Figures 9.13, 9.14 and 9.16, 9.17). This suggests the presence of a discrete protein species, at the meniscus. Table 9.10 shows the Mw and Mz values calculated at the

M_w and M_2 are the weight- and 2-average molecular weights calculated from the ends of the solution column (p. 164).

$\bar{M}_w$ and $\bar{M}_2$ are weight- and 2-average molecular weights calculated from the analysis of the data through the solution column (p. 164, 165).

$\bar{M}_{w(r)}$ is the mean of the weight-average molecular weights calculated at several points in the solution column (p. 165).

Table 9.9. Results from sedimentation equilibrium experiments of hexokinase

No	buffer	pH	[KCl] (M)	I	$Mw \times 10^3$	$Mz \times 10^3$	Mz/Mw	$\overline{Mw} \times 10^3$	$\overline{Mz} \times 10^3$	$\overline{Mw}(r) \times 10^3$
1	Phosphate	7.0	-	0.1	111.1	179.0	1.61	102.4	173.6	106.1
2	Phosphate	7.0	-	0.1	175.5	375.2	2.14	129.4	376.4	146.5
3	Phosphate	7.0	2	2.1	180.9	486.8	2.69	135.7	477.9	158.8
4	Phosphate	8.0	0.2	0.21	160.9	277.2	1.72	133.0	277.4	141.5

All solutions contained 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Experiments were performed at 10°C.

The protein concentration in experiment No 2 was 3 mg/ml. In all other experiments the protein concentration was 8 mg/ml.

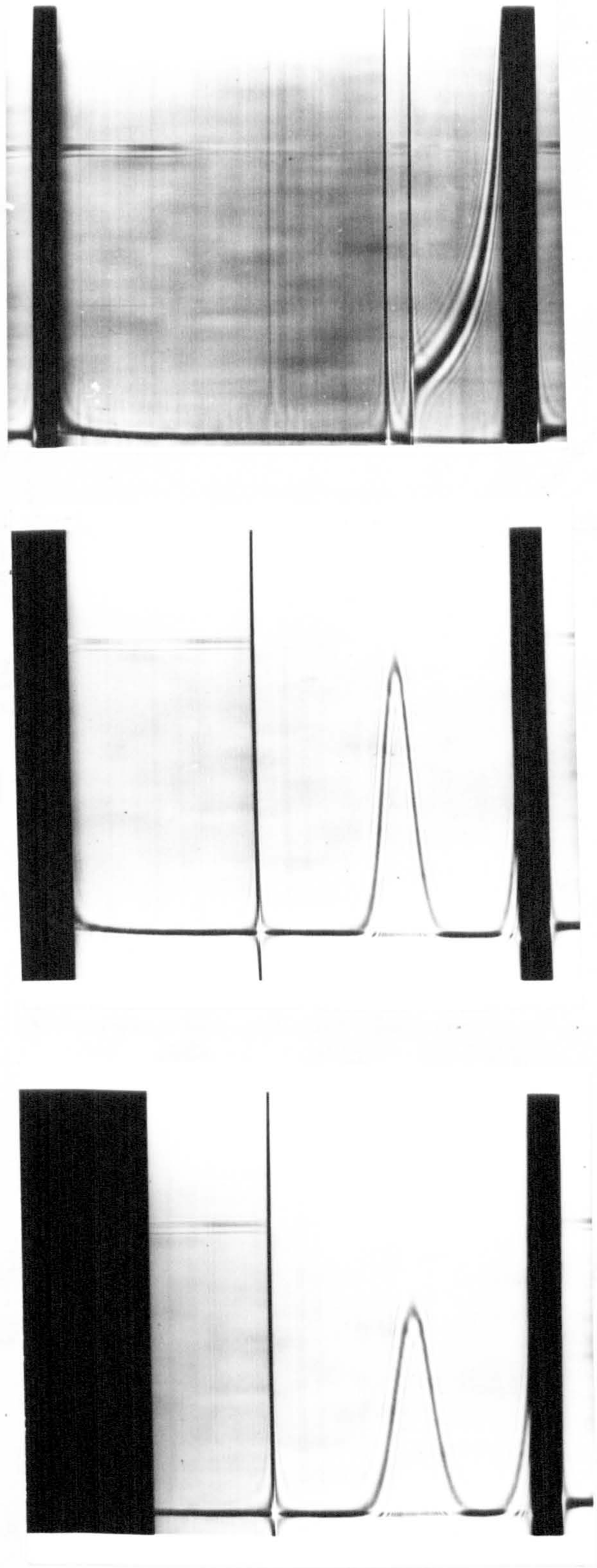
Table 9.10. Molecular weights calculated near the meniscus

Results are presented for the same sequence of sedimentation equilibrium runs as in Table 9.9. Molecular weights at the meniscus were calculated from the first six points from the meniscus.

No	Buffer	pH	KCl (M)	I	Mw x 10^3	Mz x 10^3
1	Phosphate	7.0	—	0.1	88.11	110.8
2	Phosphate	7.0	—	0.1	85.48	138.0
3	Phosphate	7.0	2	2.1	86.24	138.1
4	Tris-HCl	8.0	0.2	0.21	97.12	133.9

Figure 9.12. Sedimentation equilibrium of hexokinase in phosphate buffer pH = 7.0, I = 0.1

The top photograph shows the equilibrium pattern after centrifugation at 6,995 r.p.m. for 70 hours at 10°C. The lower photographs show the schlieren pattern at 30 and 62 minutes respectively, after boundary formation. Schlieren plate angle 60°.



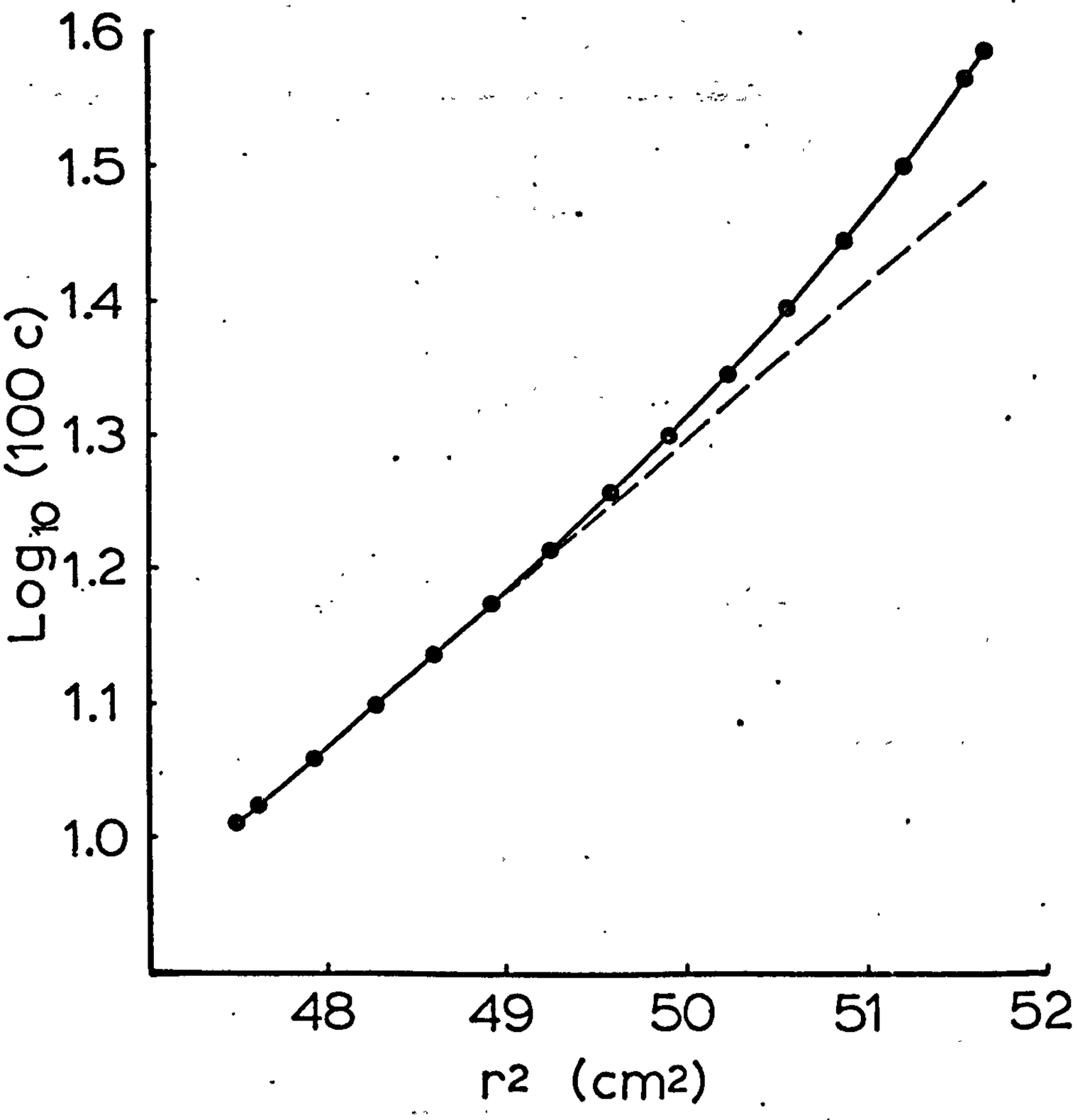


Figure 9.13. Sedimentation equilibrium of hexokinase in phosphate buffer pH = 7.0, I = 0.1

The figure shows the logarithm of the concentration plotted against the square of the radial displacement after 70 hours at 6,995 r.p.m. The temperature was $10^{o}C$.

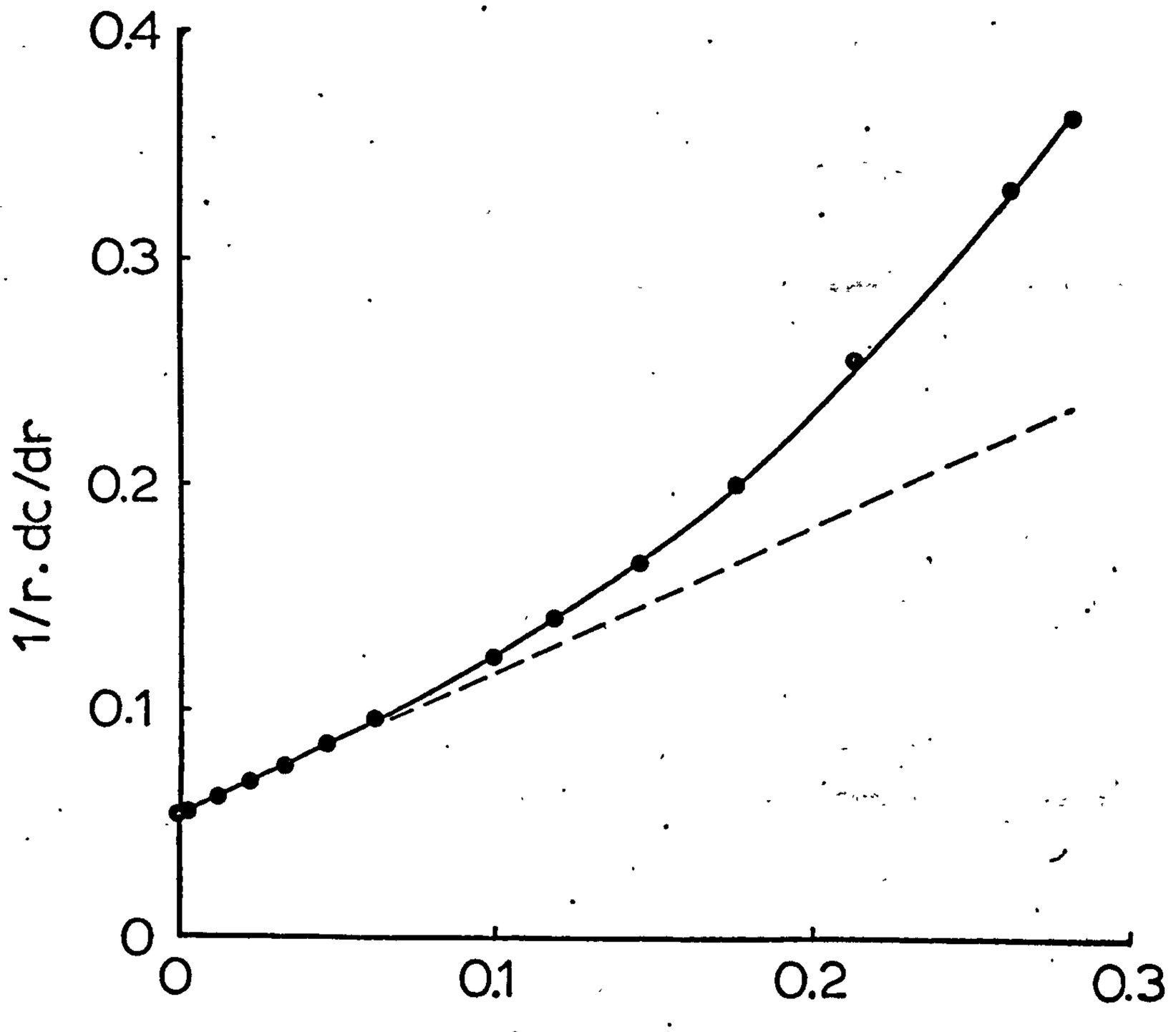
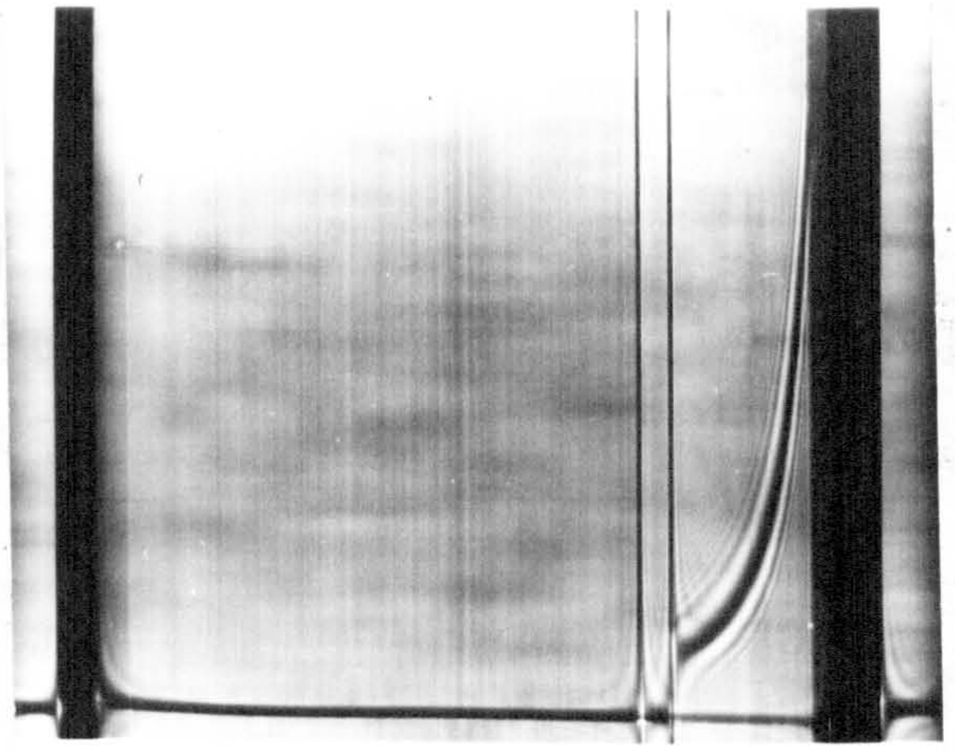


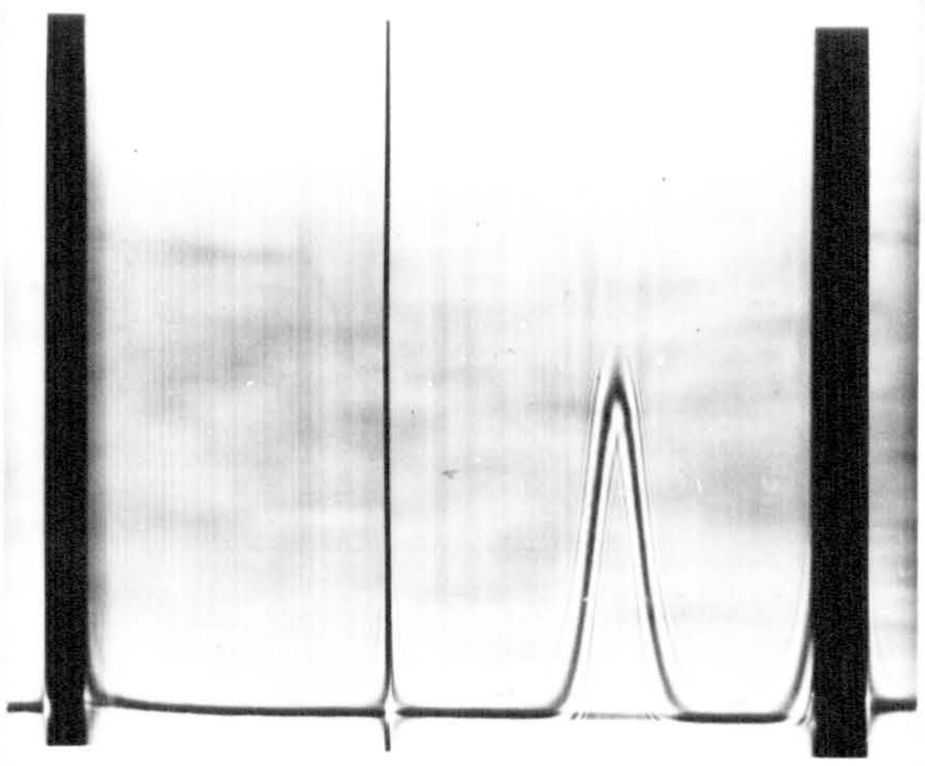
Figure 9.14. Sedimentation equilibrium of hexokinase in phosphate buffer pH = 7.0, I = 0.1.

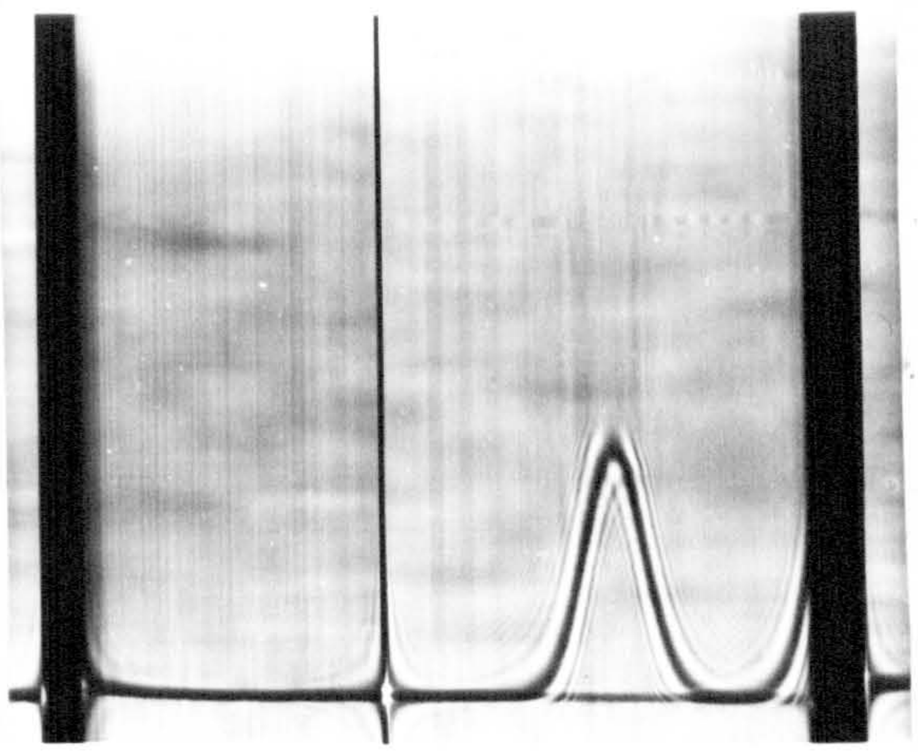
The figure shows the ratio of the concentration gradient to radial displacement versus the difference in concentration from that at the meniscus. After 70 hours at 6,995 r.p.m. and at 10°C.

Figure 9.15. Sedimentation equilibrium of hexokinase in Tris-HCl buffer pH = 8.0, I = 0.01 containing 0.2 M KCl.

The top photograph shows the equilibrium pattern after centrifugation at 6,995 r.p.m. for 70 hours at 10°C. The lower photographs show the schlieren pattern at 28 and 60 minutes respectively, after boundary formation. Schlieren plate angle 65°.







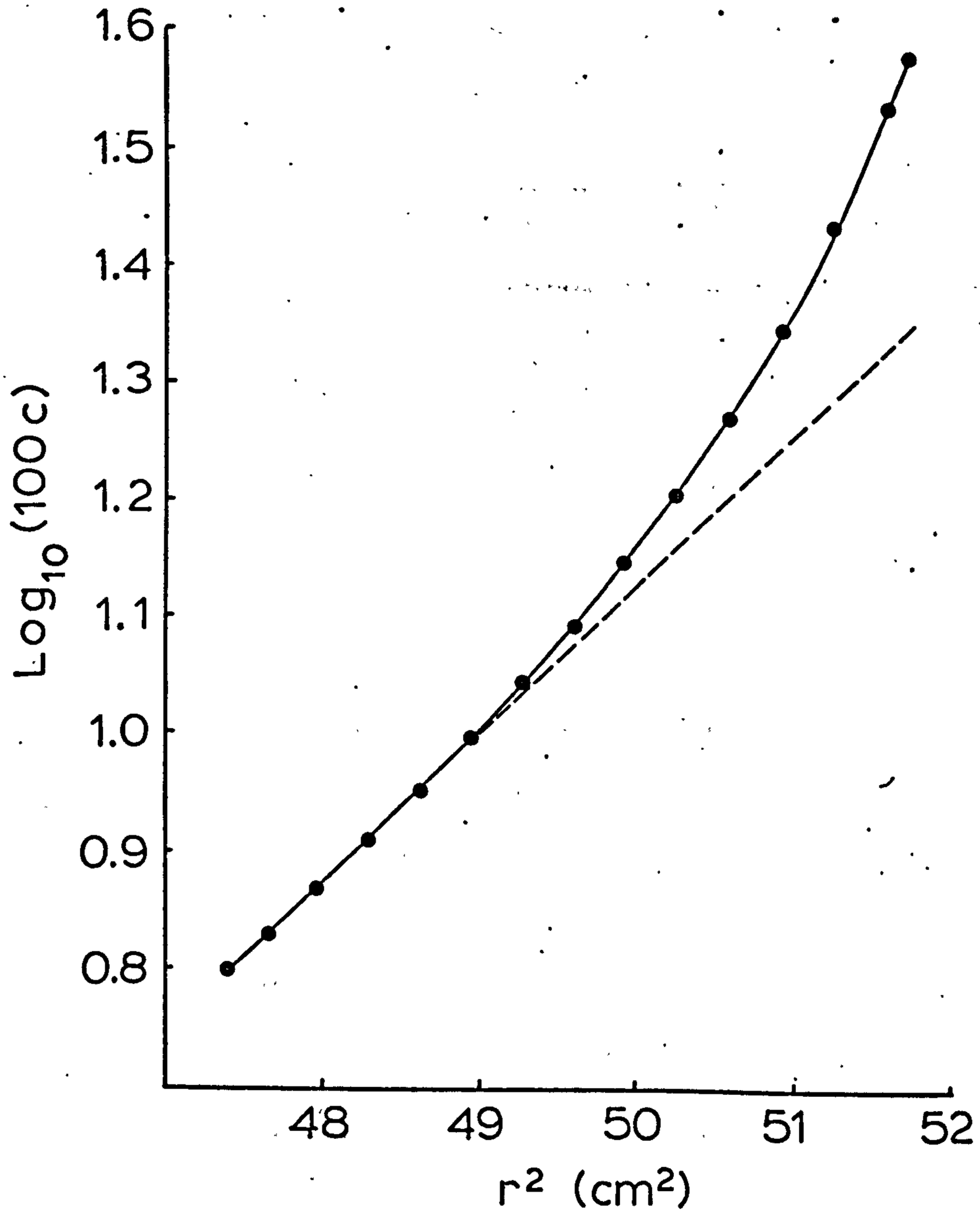


Figure 9.16. Sedimentation equilibrium of hexokinase in Tris-HCl buffer pH = 8.0, I = 0.01 + 0.2 M KCl.

The figure shows the logarithm of the concentration plotted against the square of the radial displacement after 70 hours at 6,995 r.p.m. The temperature was 10°C.

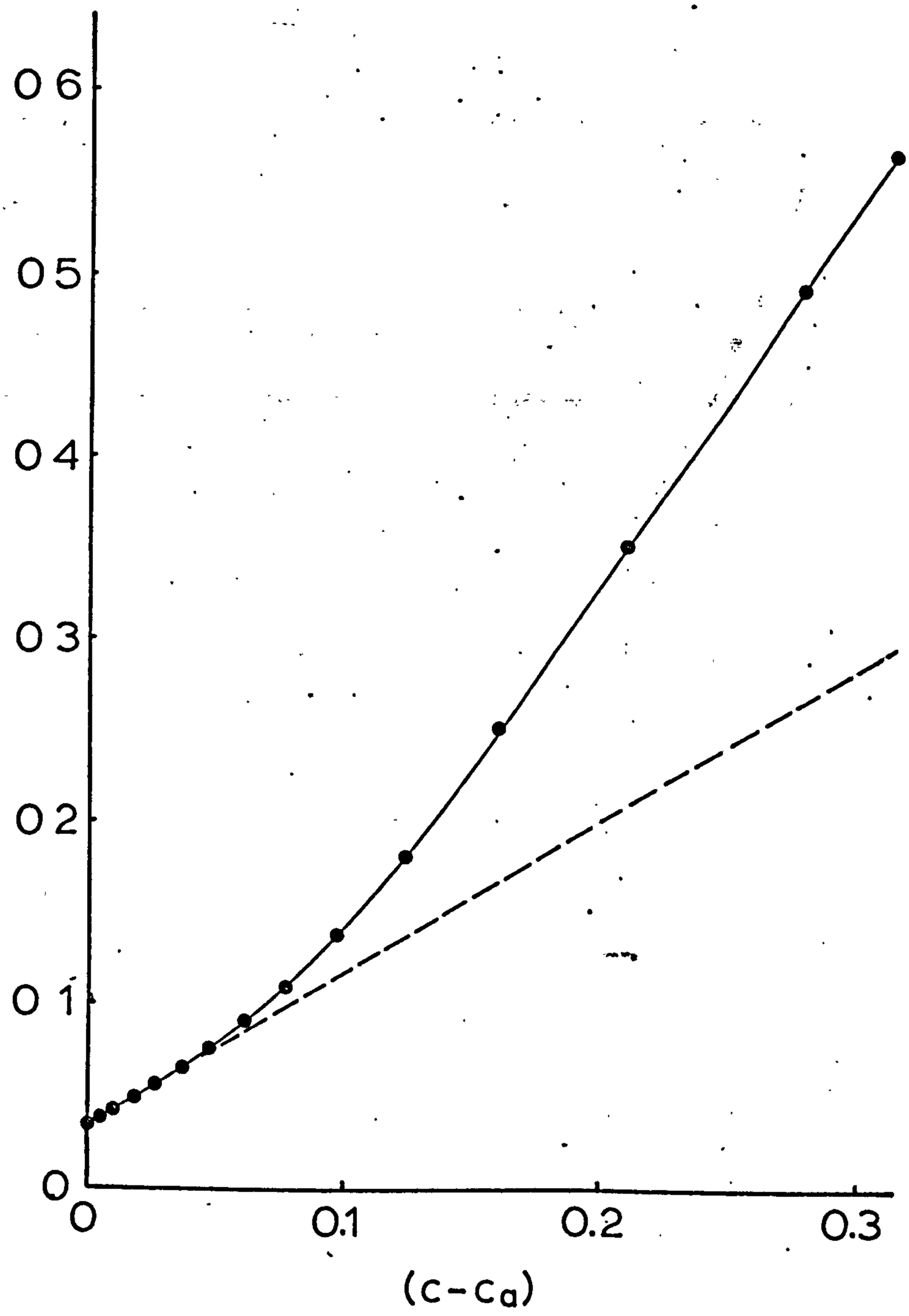


Figure 9.17. Sedimentation equilibrium of hexokinase in Tris-HCl buffer pH = 8.0, I = 0.01 + 0.2 M KCl

The figure shows the ratio of the concentration gradient to radial displacement versus the difference in concentration from that at the meniscus. After 70 hours at 6,995 r.p.m. and at 10°C.

meniscus. With the freshest material (experiment No 1), Mz at the meniscus was found equal to 110,800 near to the value obtained by gel filtration and the combined $s_{20.w}$ and $D_{20.w}$ coefficients. The lower value calculated for Mw from this experiment at the meniscus, implies the possible removal of material from the solution to the bottom of the cell. The higher Mz values at the meniscus for the other sedimentation equilibrium experiments may have been caused by the presence of dimer at the meniscus.

Size heterogeneity due to suggested hexokinase polymers was also observed for the porcine heart enzyme by Easterby (1971). Rat brain (Chou and Wilson, 1972) and rat skeletal muscle (Holroyde and Trayer, 1976) hexokinases were reported to be homogeneous by high-speed sedimentation equilibrium experiments. This method revealed homogeneity for porcine heart hexokinase whereas low speed sedimentation equilibrium for the same enzyme showed heterogeneity (Easterby, 1971).

(c) Glucose 6-phosphate mediated dimerization

(1) Resolution of two sedimentation boundaries

The effect of glucose 6-phosphate on the sedimentation pattern of human heart hexokinase is shown in Figures 9.18 to 9.20.

The appearance of two sedimentation boundaries is clearly seen in the presence of glucose 6-phosphate. The slower one corresponded to the single boundary observed in the absence of glucose 6-phosphate, while the faster one corresponded to a sedimentation coefficient of 7.4 to 8.2 depending on buffer conditions.

The increase in sedimentation of the faster boundary is difficult to explain by a change of the frictional ratio alone. Therefore dimerization is suggested. The ratio of sedimentation coefficients of the monomer, calculated in the absence of glucose 6-phosphate, and the dimer calculated in the presence of this ligand was equal to 1.43 (in Tris-HCl buffer pH = 8.0, I = 0.01 + 0.2 M KCl; protein concentration 8 mg/ml) and 1.48 (in phosphate buffer pH = 7.0, I = 0.1; protein concentration 4.5 - 6.5 mg/ml). These ratios are less and near to the expected ratio of 1.59, for a monomer-dimer relation with no change in the frictional coefficient occurring upon dimerization.

The resolution of two sedimentation boundaries for a monomer-dimer equilibrium may be caused either by a slow equilibrium, as compared to the time of centrifugation, (whereas for a fast equilibrium a non-symmetric peak is predicted; Gilbert, 1955), or by a fast equilibrium when a concentration gradient of unbound ligand (mediating dimerization) is maintained in the ultracentrifuge cell,

due to differential transport of monomer and dimer (Cann and Goad, 1970a, 1970b).

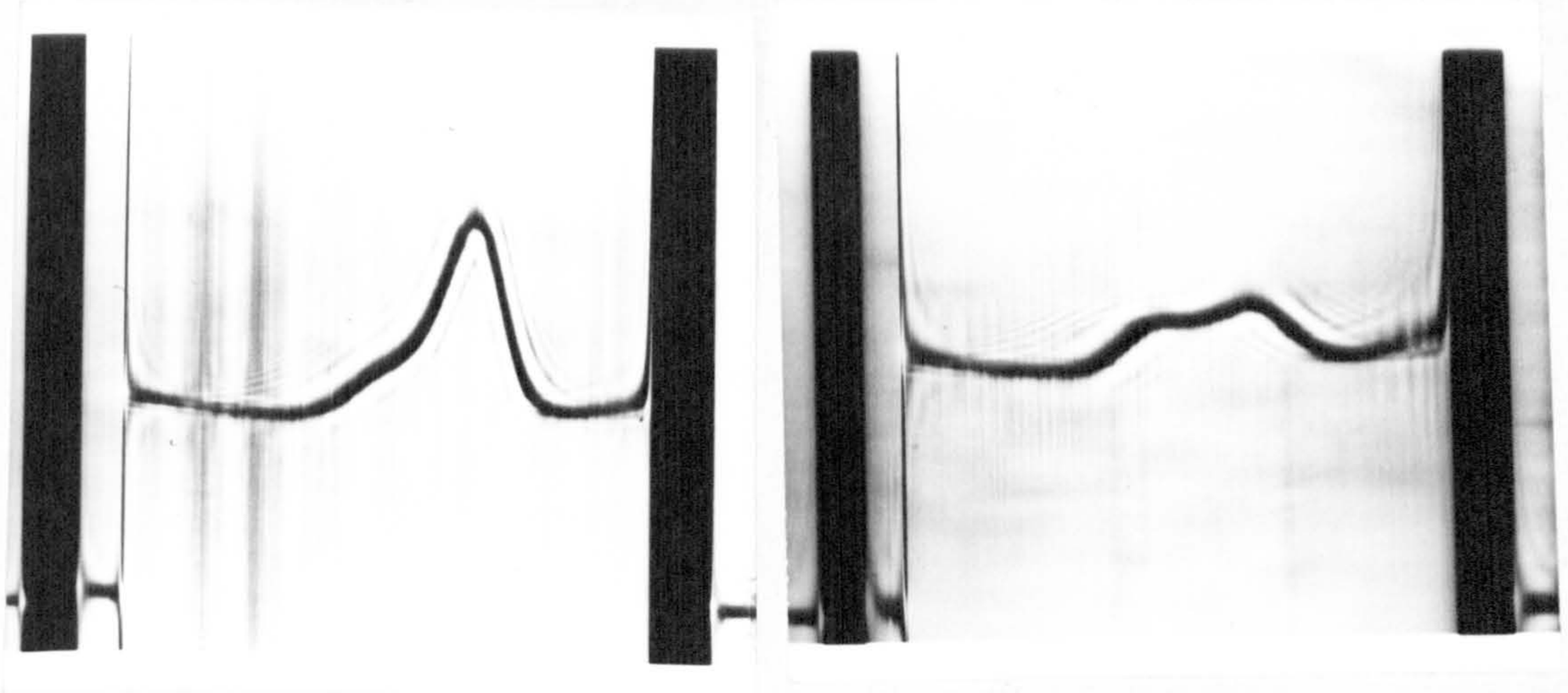
The effect of glucose 6-phosphate in dimerization of hexokinase has been reported for bovine brain (Chakrabarti and Kenkare, 1974) and porcine heart hexokinase (Easterby, 1975). The latter author reported evidence for the existence of the monomer-dimer equilibrium even in the absence of glucose 6-phosphate but in favour of monomer.

(2) <u>Sedimentation pattern at different protein and glucose 6-phosphate concentrations</u>

If the protein concentration was lowered from 9 to 4.5 mg/ml, in the presence of 1 mM glucose 6-phosphate, the area under the faster boundary became less in favour of the slower one, whereas the sedimentation coefficient of the faster boundary did not change (Figure 9.18).

If the glucose 6-phosphate concentration was increased from 50 μM to 1 mM, at a protein concentration of 4.5 mg/ml, the area under the faster boundary increased (Figure 9.19).

Dimerization was not complete even at saturating glucose 6-phosphate concentration. This suggests the existence of at least two distinct equilibria, one for monomer and the ligand and the other for ligand bound monomer and dimer. The smaller degree of dimerization at 50 μM glucose 6-phosphate may be explained by the low Ki of mammal-



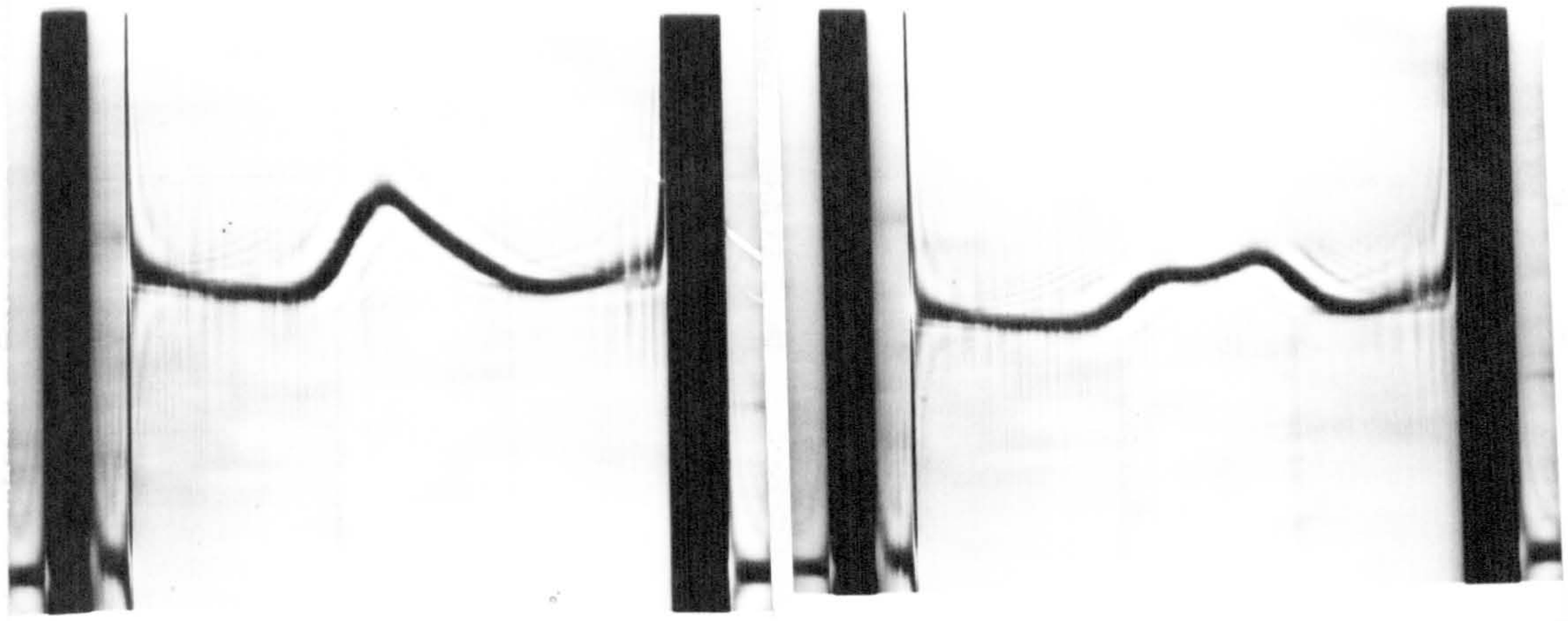
(A) (B)

Figure 9.18. Sedimentation pattern of hexokinase in the presence of 1 mM glucose 6-phosphate at two protein concentrations

(A) : Protein concentration 9 mg/ml (—, 7.40 S)
(B): : Protein concentration 4.5 mg/ml (—, 7.45 S)

The buffer was Tris-HCl pH = 8.0, I =0.01 containing 0.2 M KCl, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 1 mM glucose 6-phosphate. Photographs were taken 90 minutes after reaching the speed of 59,780 r.p.m. at schlieren plate angles of (A) 55°, (B) 45°. The temperature was 10°C.

Values inside the brackets represent the sedimentation coefficients, $s_{20.w}$, for the slow and fast sedimentation boundaries, while dashes inside brackets mean insufficient resolution for the estimation of the respective sedimentation coefficient.



(A) (B)

Figure 9.19. Sedimentation pattern of hexokinase at two glucose 6-phosphate concentrations

(A) : 50 μM glucose 6-phosphate (5.40 S, —)

(B) : 1 mM glucose 6-phosphate (—, 7.45 S)

The buffer was Tris-HCl pH = 8.0, I = 0.01 containing 0.2 M KCl, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Photographs were taken 90 minutes after reacing the speed of 59,780 r.p.m. at schlieren plate angles of (A) 40^{o}, (B) 45^{o}. The protein concentration was 4.5 mg/ml and the temperature was 10^{o}C.

ian hexokinases for this ligand i.e. 50 μM for glucose 6-phosphate versus fructose for porcine heart hexokinase (Easterby and O'Brien, 1973) and 10.8 μM for glucose 6-phosphate versus $MgATP^{2-}$ for human erythrocyte hexokinase (Rijksen and Staal, 1977).

Chakrabarti and Kenkare (1974) reported complete dimerization of bovine brain hexokinase at 100 μM glucose 6-phosphate whereas at 10 μM glucose 6-phosphate 50% dimerization occurred. They also observed that by lowering protein concentration dimerization diminished and at 1 mg/ml protein concentration no noticeable difference between the sedimentation behaviour of the enzyme in the presence and absence of glucose 6-phosphate was found.

Easterby (1975) reported only 50% dimerization even at saturating glucose 6-phosphate concentration (1 mM). Less dimerization was observed at 20 μM glucose 6-phosphate.

(3) <u>Reversibility of glucose 6-phosphate mediated dimerization</u>

The effect of glucose 6-phosphate was reversible as shown by restoring the initial sedimentation velocity after removal of the ligand by dialysis (Figure 9.20). The same phenomenon was observed for bovine brain and porcine heart hexokinase (Chakrabarti and Kenkare, 1974;

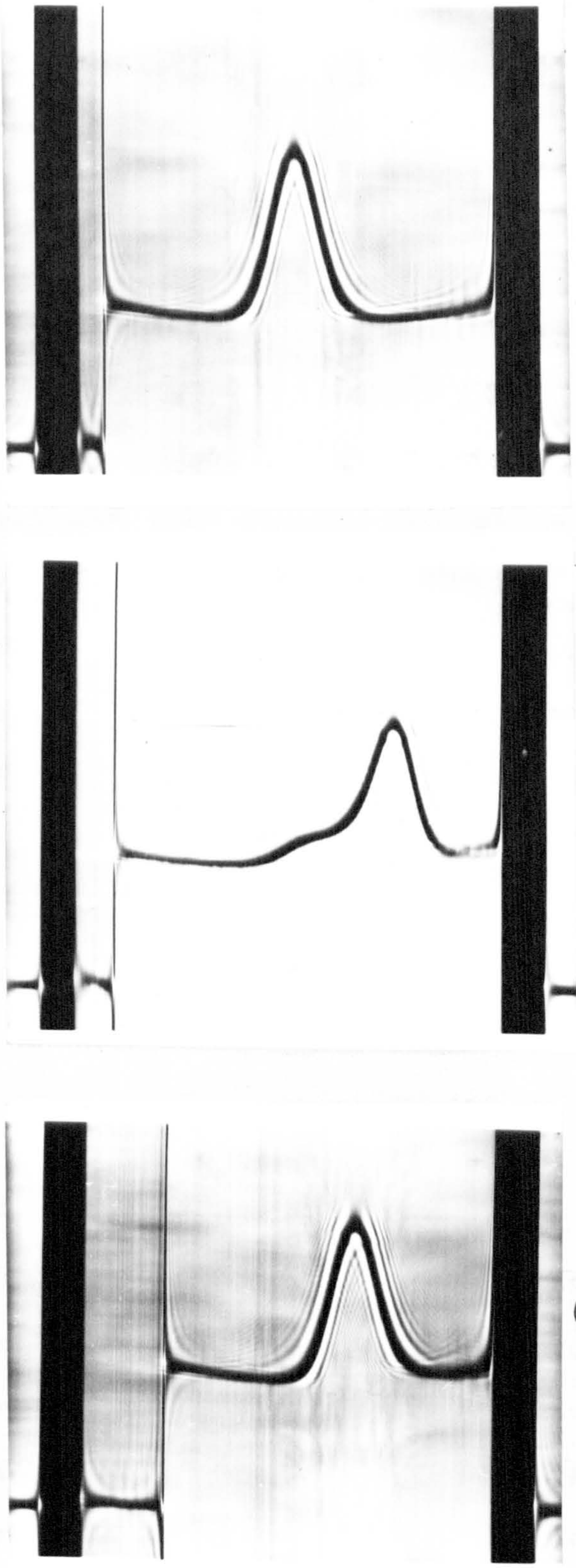
Figure 9.20. The reversibility of the effect of glucose 6-phosphate on the sedimentation pattern of hexokinase

(A) : Absence of glucose 6-phosphate (5.60 S)

(B) : Sample (A) after dialysis against 1 mM glucose 6-phosphate (—, 8.20 S)

(C) : Sample (B) after removal of glucose 6-phosphate by dialysis (5.50 S)

The buffer was phosphate pH = 7.0, I = 0.1 containing 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Photographs were taken 90 minutes after reaching the speed of 59,780 r.p.m. at schlieren plate angle of 55^{o}. The protein concentration was 8 mg/ml and the temperature 10^{o}C.



(A)

(B)

(C)

Easterby, 1975). The reversibility of the dimerization mediated by glucose 6-phosphate is an obvious prerequisite for the suggested monomer-dimer equilibrium.

However if human heart hexokinase at a concentration of 4.5 mg/ml was dialysed against acetate buffer pH = 6.0, I = 0.1 containing 0.2 M KCl, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 1 mM glucose 6-phosphate, a heavy precipitate was developed suggesting extensive polymerization under these conditions. If this suspension was dialysed against Tris-HCl buffer pH = 8.0, I = 0.01 containing 0.2 M KCl, 10 mM glucose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol, the precipitate redissolved and upon ultracentrifugation a pattern similar to that of hexokinase in the absence of glucose 6-phosphate was observed (Figure 9.21), thus indicating the reversibility of this effect.

(4) Effect of fructose 6-phosphate

Fructose 6-phosphate at a concentration of 5 mM was found to promote dimerization (Figure 9.22). However this ligand was not found to promote dimerization with bovine brain (Chakrabarti and Kenkare, 1974) and porcine heart (Easterby, 1975) hexokinases even at a concentration of 10 mM of fructose 6-phosphate for the latter enzyme.

One possibility is that fructose 6-phosphate used in

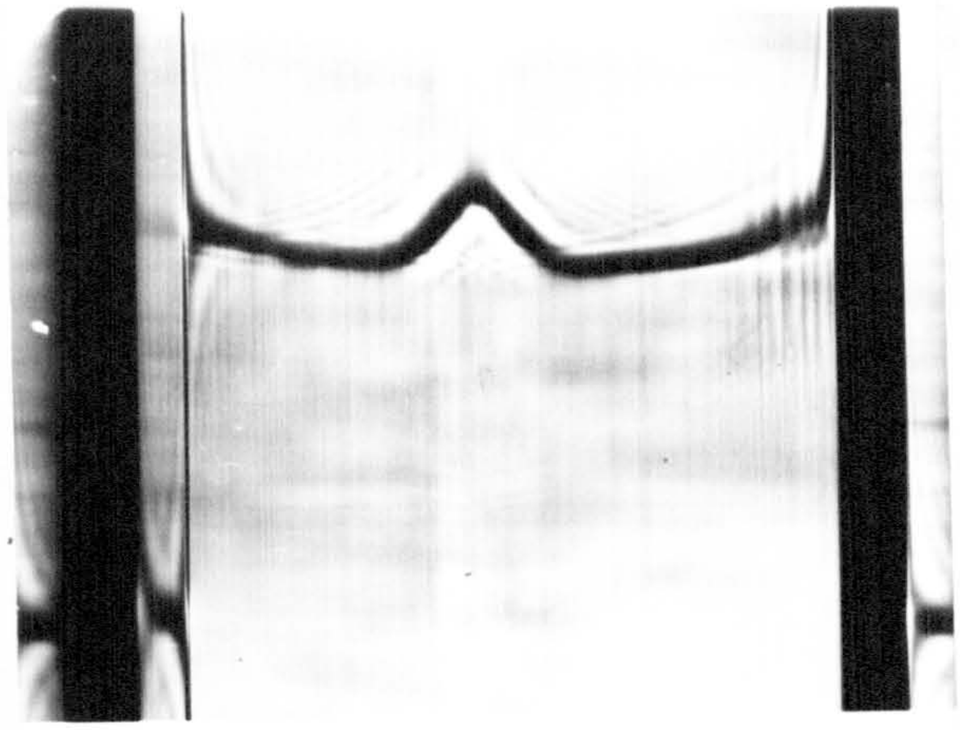


Figure 9.21. *Sedimentation pattern of redissolved hexokinase after precipitation by glucose 6-phosphate at pH=6.0.*

Buffer conditions are described in text. The protein concentration was 2 - 3 mg/ml. The schlieren plate angle was 40° (5.40S). Photographs were taken 90 minutes after reaching the speed of 59,780 r.p.m. The temperature was 10°C.

Tris-HCl buffer pH=8.0, I=0.01+0.2M KCl containing 10mM glucose, 1mM EDTA and 0.1%. (v/v) 2-mercaptoethanol.

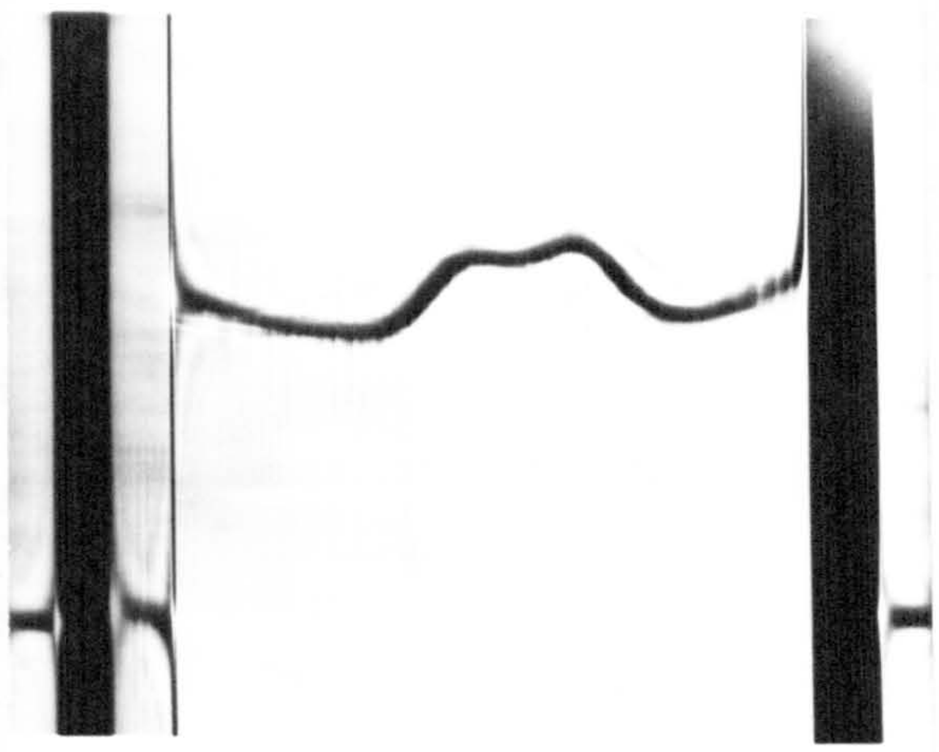


Figure 9.22. Effect of fructose 6-phosphate on sedimentation pattern of hexokinase

The buffer was Tris-HCl pH = 8.0, I = 0.01 containing 10 mM glucose, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 5 mM fructose 6-phosphate. The protein concentration was 4.5 mg/ml and the schlieren plate angle 45°, (5.20 S, 7.35 S).

Photographs were taken 90 minutes after reaching the speed of 59,780 r.p.m. The temperature was 10°C.

this experiment was contaminated with glucose 6-phosphate. Commercial preparations of fructose 6-phosphate by Boehringer and Sigma are reported by the manufacturers to contain less than 2% and less than 1% glucose 6-phosphate respectively.

Another possibility is that fructose 6-phosphate binds at the same hexokinase site as glucose 6-phosphate although with less affinity promoting the needed conformational change for dimerization. Fructose 6-phosphate is generally accepted not to be an inhibitor of mammalian hexokinase including the human enzyme (de Verdier and Garby, 1965; Gerber et al., 1974). Recently however, Rijksen and Staal (1977) reported that fructose 6-phosphate was inhibiting human erythrocyte hexokinase competitively with respect to MgATP with Ki = 0.16 mM. It is possible therefore that fructose 6-phosphate mediated inhibition and dimerisation are properties peculiar to human hexokinase.

(5) Effect of other ligands on the glucose 6-phosphate promoted dimerization

(i) Glucose

Glucose at 10 mM final concentration had no obvious effect either promoting or releasing glucose 6-phosphate mediated dimerization, as shown in Figure 9.23.

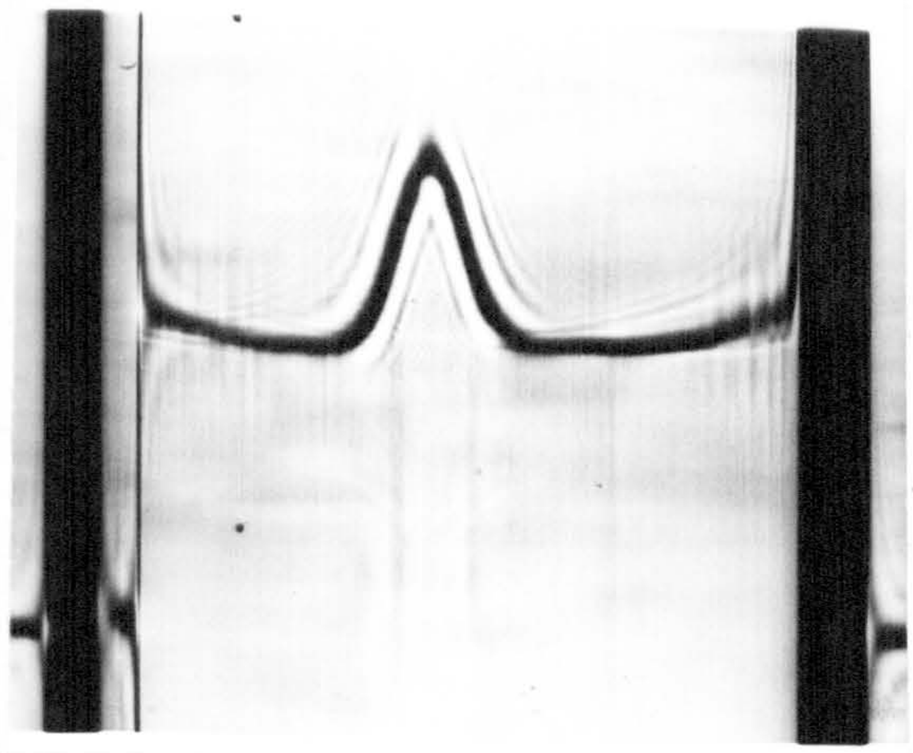
Figure 9.23. The effect of glucose 6-phosphate on the sedimentation pattern of hexokinase in the presence and absence of glucose

(A) : 10 mM glucose, no glucose 6-phosphate (5.20 S)

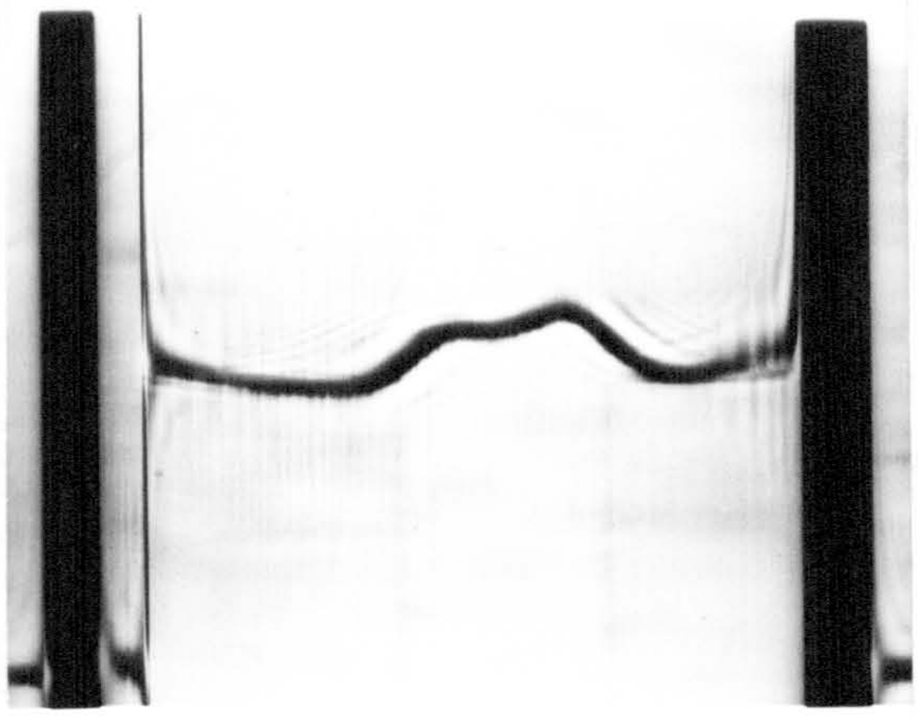
(B) : No glucose, 1 mM glucose 6-phosphate (—, 7.45 S)

(C) : 10 mM glucose, 1 mM glucose 6-phosphate (—, 7.50 S)

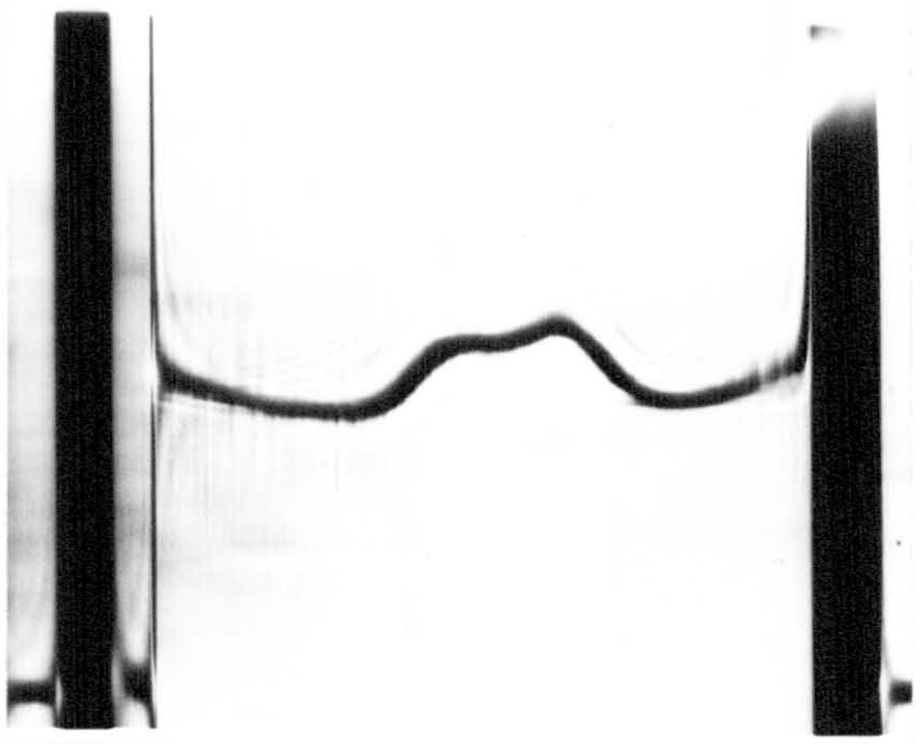
The buffer was Tris-HCl pH = 8.0, I = 0.01 containing 0.2 M KCl, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Photographs were taken 90 minutes after reaching the speed of 59,780 r.p.m. at a schlieren plate angle of 45°. The protein concentration was 4.5 mg/ml and the temperature 10°C.



(A)



(B)



(C)

This finding is in accordance with similar observations for bovine brain (Chakrabarti and Kenkare, 1974) and porcine heart hexokinase (Easterby, 1975). It is also in accordance with kinetic observations for non-competitive inhibition of human erythrocyte hexokinase by glucose 6-phosphate versus glucose (Gerber et al., 1974; Rijksen and Staal, 1977).

(ii) $\underline{MgATP^{2-}}$

1 mM $MgCl_2$ did not change the sedimentation pattern of human heart hexokinase (Figure 9.24 B). However in the presence of 2 mM $MgCl_2$ and 1 mM ATP a small dimerization was observed (Figure 9.24 C). Therefore it seems that $MgATP^{2-}$ can bind to hexokinase in the absence of glucose. Dimerization persisted in the presence of 2 mM $MgCl_2$, 1 mM ATP and 1 mM glucose 6-phosphate (9.24 D).

For bovine brain hexokinase (Chakrabarti and Kenkare, 1974) ATP at a concentration of 5 mM, in the presence of 0.1 mM glucose 6-phosphate, reversed the sedimentation pattern to that of the monomer. For porcine heart hexokinase (Easterby, 1975), at 50 µM glucose 6-phosphate, ATP up to a concentration of 10 mM slightly abolished dimerization whereas 1 mM ATP and 2 mM $MgCl_2$ almost completely abolished dimerization. The effect of MgATP was found to be competitive with respect to glucose 6-phosphate.

Figure 9.24. The effect of Mg^{2+}, $MgATP^{2-}$ and glucose 6-phosphate plus $MgATP^{2-}$ on the sedimentation pattern of hexokinase

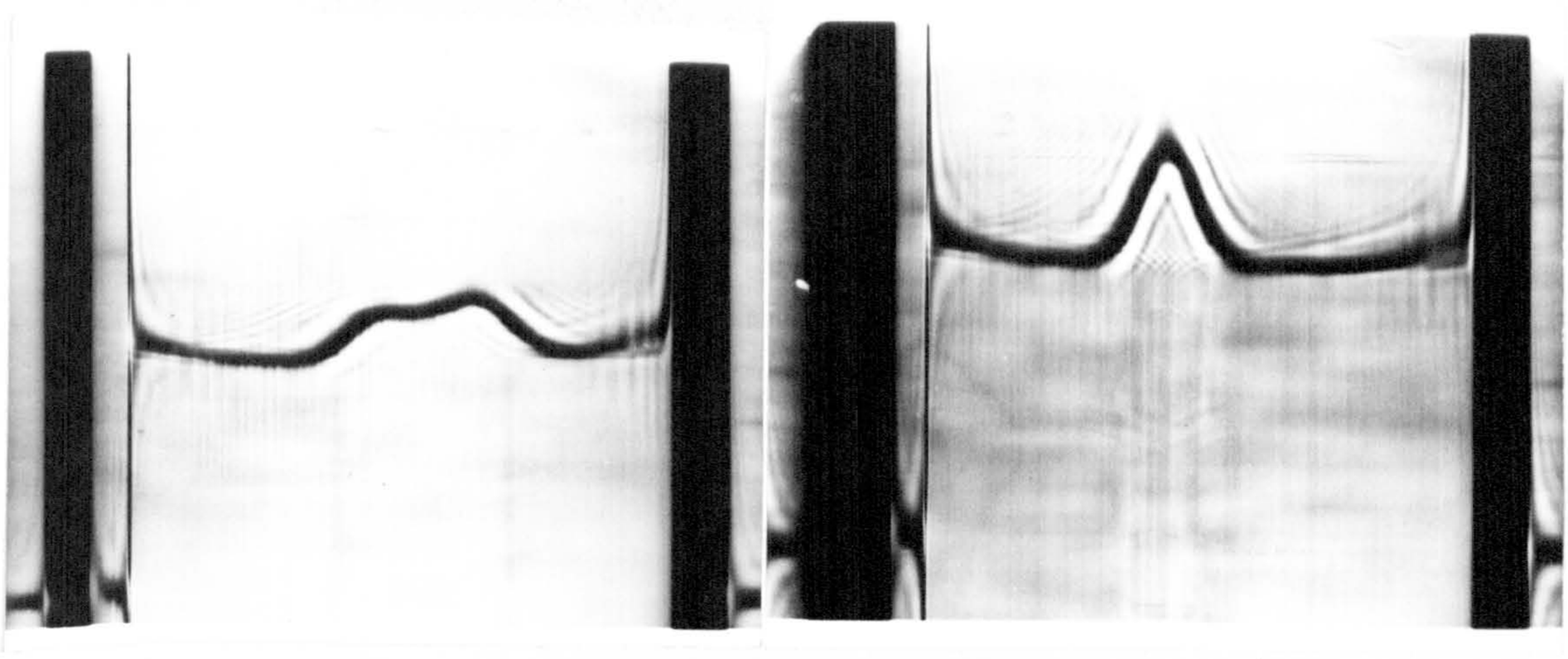
(A) : 1 mM glucose 6-phosphate (—, 7.45 S)

(B) : 1 mM $MgCl_2$ (5.30 S)

(C) : 2 mM $MgCl_2$ and 1 mM ATP (5.30 S, 8.00 S)

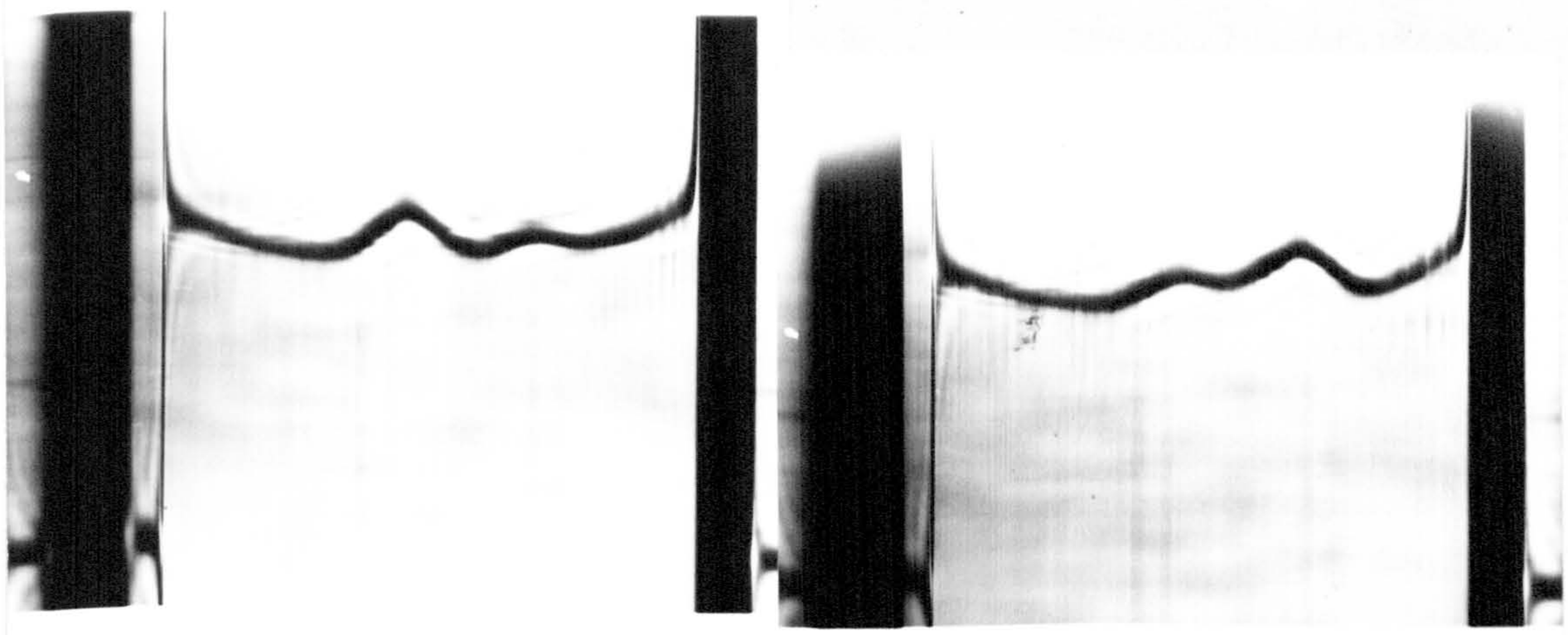
(D) : 2 mM $MgCl_2$, 1 mM ATP and 1 mM glucose 6-phosphate (5.55 S, 7.90 S).

The buffer was Tris-HCl pH = 8.0, I = 0.01 containing 0.2 M KCl, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Photographs were taken 90 minutes after reaching the speed of 59,780 r.p.m. at schlieren plate angle of (A) 45°, (B) 40°, (C) 40° (D) 40°. The protein concentration was 4.5 mg/ml and the temperature 10°C.



(A)

(B)



(C)

(D)

However, both reports for bovine brain and porcine heart hexokinase do not mention sedimentation experiments with $MgATP^{2-}$ alone i.e. in the absence of glucose 6-phosphate.

(iii) Pi

Comparison of Figures 9.18A and 9.20B i.e. effect of 1 mM glucose 6-phosphate in Tris-HCl buffer pH = 8.0, I = 0.01 + 0.2 M KCl and in phosphate buffer pH = 7.0, I = 0.1 (47 mM Pi) shows no significant differences in sedimentation patterns. Therefore no significant effect of Pi is suggested at 1 mM glucose 6-phosphate concentration.

For bovine brain hexokinase (Chakrabarti and Kenkare, 1974) 10 mM Pi reverted the enzyme to its normal sedimentation behaviour in the presence of 0.1 mM glucose 6-phosphate. For porcine heart hexokinase (Easterby, 1975) 10 mM potassium phosphate did not appreciably affect the dimerization promoted by 1 mM glucose 6-phosphate, whereas when glucose 6-phosphate concentration was lowered to 50 μM the proportions changed in favour of the monomer.

(6) Comments

Two questions arise: the underlying mechanism for polymerization and the physiological significance of this phenomenon. Although complete answers must await further

investigation, certain comments can be made for the human heart enzyme.

Sedimentation equilibrium experiments showed a tendency for polymerization in the presence of glucose under the experimental conditions used. Extensive and reversible dimerization was observed by sedimentation velocity experiments in the presence of glucose 6-phosphate. Dimerization was also promoted possibly by fructose 6-phosphate and to a lesser extent by $MgATP^{2-}$.

Hexokinase may exist in an equilibrium between two conformations, the dominant one having less tendency to dimerize. The effect of the above ligands is to shift the equilibrium to the other conformation with concomitant dimer formation.

Glucose 6-phosphate does not bind at the glucose binding site, since glucose does not affect the dimerization produced by glucose 6-phosphate. Thus, since glucose binds at the active site, the binding of glucose 6-phosphate could occur at an allosteric site. It has been suggested that low Km hexokinases have evolved by gene duplication of an ancestral glucokinase-like gene (Easterby, 1971). On the basis of one glucose binding site (Ellison et al., 1974), the other initial active site may have been transformed to an allosteric site. However kinetic observations of human erythrocyte hexokinase show competitive

inhibition of glucose 6-phosphate against $MgATP^{2-}$ and non competitive against glucose (Gerber et al., 1974; Rijksen and Staal, 1977), thus implying overlapping of the glucose 6-phosphate binding site with that of MgATP in the active site. The conformational change, and dimerization, of hexokinase may arise from binding of glucose 6-phosphate to the MgATP site, or to another, allosteric site.

MgATP (in the absence of glucose) promoted some dimerization of hexokinase. Therefore, either binding at the active site influences the conformational equilibrium or the allosteric site binds $MgATP^{2-}$ as well as glucose 6-phosphate. The effect of MgATP on the enzyme also implies that it can bind in the absence of glucose according to a random mechanism (e.g. for the human erythrocyte enzyme; Gerber et al., 1973).

The dimerization by glucose 6-phosphate was observed for the human erythrocyte enzyme (Chapter 8) as well as for the heart enzyme. Since hexokinases from bovine brain (Chakrabarti and Kenkare, 1974) and procine heart (Easterby, 1975) also dimerize in the presence of glucose 6-phosphate, this phenomenon appears to be a common property of the enzyme from mammalian sources.

The present experiments also indicate that fructose 6-phosphate, in high concentration, can also promote dimer-

ization of the enzyme. Presumably this metabolite can bind at the same location as glucose 6-phosphate.

The process of dimerization does not involve the formation of disulfide bonds since it was observed in the presence of 2-mercaptoethanol. Although dimerization persisted at 0.3 ionic strength, ionic interactions can not be excluded since glucose 6-phosphate also promoted a pH-dependent precipitation of the enzyme. The increase in sedimentation coefficient of the native enzyme at pH = 6.0 suggests some association even in the absence of glucose 6-phosphate.

Although it was observed in physiological concentration of glucose 6-phosphate, dimerization may not occur at the enzyme concentration found inside the human cell. There appeared to be less dimer formation by glucose 6-phosphate when the protein concentration was decreased (Figure 9.18).

Although dimerization may not have a direct physiological role, it reflects the conformational change produced by glucose 6-phosphate. This conformational change may be an essential property in the control of hexokinase (and hence the glycolytic flux), by inhibition and alteration in the subcellular distribution of the enzyme.

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